IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	§	
	§	
YORAM REITER	§	
	§	
Serial No.: 10/073,300	§	
	§	
Filed: 02/13/2002	§	Group Art Unit: 1644
	§	
For: SINGLE CHAIN CLASS I...	§	
	§	Attorney
	§	Docket: 02/23339
Examiner: Vandervegt, Francois P	§	

Commissioner of Patents and Trademarks
P. O. Box 1450 Alexandria VA 22313

DECLARATION OF YORAM REITER UNDER 37 CFR 1.132

I am presently employed as researcher at the Technion Institute of Technology, Haifa Israel, Department of Biology, where I am a professor of Molecular Immunology and head of the laboratory of molecular immunology. I received my Ph.D. degree from the Weizmann Institute of Science in 1993, worked as a post-doctoral fellow in the laboratory of molecular biology at the National Cancer Institute, National Institutes of health in Bethesda Maryland where I developed new approach to targeted therapy of cancer.

My research focuses on molecular immunology of cancer with the emphasis of developing new antibody-based molecules for targeted therapy of cancer. Since the beginning of my career, I have published 70 scientific articles in highly regarded journals and books, and have presented my achievements at many international scientific conferences.

I am a member of the American Association of Immunologists, the American Association for Cancer Research, the Israel Immunology Society, and was awarded several research prizes including the US government technology transfer award, the Rothschild Foundation post doctoral award, the Alon fellowship award for outstanding young scientist administered by the Israel council for higher education, the Teva Prize for research, The Yuludan and Taub prizes for excellence in research.

I was elected recently to the editorial board of pharmaceutical design and serve as a reviewer for many journals such as the Journal of Immunology, Cancer Research, Journal of Immunological Methods and more.

I am a co-inventor of the subject matter claimed in the above-referenced U.S. patent application.

I have read the Official actions issued with respect to the above-identified application.

In this Official action, the Examiner has rejected claims 1-3 under 35 U.S.C. § 102(b) as being anticipated by Mottez et al [J. Exp. Med (1995) 181: 493-502].

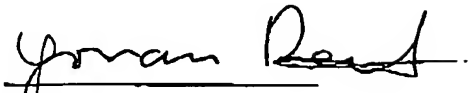
Attached is a manuscript of a recent study performed in my laboratory in which we applied the teachings of the instant application towards generation of a chimeric polypeptide which includes an antigenic peptide covalently attached to the N terminus of a single-chain β-2-microglobulin-HLA-A2 heavy chain fusion protein. The antigenic peptides used in this study were G9-209M, G9-280V, and $MART_{27-35}$ which are derived from melanoma differentiation antigens gp100 and MART1 and the EBV-derived peptide $GLC_{280-288}$. Each antigenic peptide was fused to the N-terminus of the human β_2-microglobulin gene through a 15-amino acid long flexible linker [$(Gly4\text{-}Ser)_3$]. These chimeric proteins were purified from inclusion bodies by cell disruption followed by solubilization in the presence of Guanidine HCl and refolding in the presence of a redox-shuffling buffer system.

This study conclusively shows that the chimeric proteins made according to the teachings of the present invention were correctly folded and capable of producing an appreciable specific T-cell re-activation. Such soluble human MHC molecules with covalently linked peptides offer improved molecular tools over murine MHC complexes, not only for molecular studies but also for generating new fusion molecules for immunotherapeutic approaches.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United states Code and that such willful false statements may jeopardize the validity of the application or any patent issued

thereon.

21 December, 2004



Enc.:

CV of Yoram Reiter and a recent paper by Oved et al.

CURRICULUM VITAE (November 2004)

Name: Yoram Reiter

Date and place of Birth: October 25, 1959, Haifa, Israel

Citizenship: Israel

Marital Status: Married, two children

Education:

August 1984	B.Sc Biochemistry, Tel Aviv University, Faculty of Life Sciences, Tel Aviv, Israel.
February 1987	M.Sc Department of Chemical Immunology, The Feinberg Graduate School of The Weizmann Institute of Science, Rehovot, Israel. (Thesis: Immunotargeting of complement to tumor cells by monoclonal antibody-complement conjugates)
January 1993	Ph.D., Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel. (Thesis: Molecular mechanisms of tumor cell resistance to complement-mediated immune damage; under the guidance of Prof. Zvi Fishelson)

Brief Chronology of Employment:

1/1993-7/1997 Visiting Fellow, Laboratory of Molecular Biology,
Division of Basic Sciences, National Cancer Institute
National Institutes of Health, Bethesda MD.

8/1997-8/1998 Senior Scientist, Peptor Ltd, Weizmann Science Park
Rehovot, Israel.

9/1998-6/2003 Senior Lecturer (Assistant Professor)
Faculty of Biology
Technion-Israel Institute of Technology
Haifa, Israel.

7/2003-present Associate Professor (Tenured)
Faculty of Biology
Technion-Israel Institute of Technology
Haifa, Israel.

Military Service:

1978-1981 Israel Defence Forces

Prizes, awards, and scholarships:

1988-1991 Ph.D. Studentship Scholarship. The Wolfson Foundation at the Weizmann Institute of Science.

1989 FEBS (Federation of European Biomedical Societies) Fellowship for graduate students.

1991 FEBS (Federation of European Biomedical Societies) Fellowship for graduate students.

1992-1993 The Rothschild Postdoctoral Fellowship Award for outstanding Ph.D. graduates.

1994 U.S. Federal Technology Transfer Award, for an outstanding scientific

contribution of value to the USA. Awarded by the National Cancer Institute, National Institutes of Health, Bethesda MD.

1998-2001 The "Alon" Fellowship Award, for outstanding young scientists. Awarded by the Israel Council For Higher Education, The Israel Ministry of Education ("VATAT").

1999 Award- The L. Naftali Science Foundation for Biology and Medicine, Jerusalem, Israel.

1999-2001 The Leah and Donald Lewis Academic Lectureship award administrated
By the Technion-Israel Institute of Technology.

1999-2002 The TEVA Fellowship Award for Young Scientists in Life Sciences and Medicine. Awarded by TEVA Pharmaceutical Industries Ltd. Israel.

1999-2003 Research Career Development Award (RCDA), ICRF-Israel Cancer Research Fund (USA) New-York, USA.

2000-2001 The TEVA RESAERCH GRANT AWARD , Awarded through The Israel Academy of Sciences and Humanities by TEVA Pharmaceutical
Industries Ltd. Israel.

2003 Citation for excellence in teaching – Center for Promotion of Teaching-Technion

2003 The Henry Taub Prize for Excellence in Research, Awarded by the Technion Board of Governs.

2004 The Juludan Prize for Application of science and technology in medicine, Awarded by the Technion.

Invited Lectures:

1) **Invited speaker**, The 5th Meeting on the Molecular Basis of Cancer. Foundation for Advanced Cancer Studies, Frederick, MD,USA, June 1994.

Title: Recombinant Antibodies for Cancer Therapy

2) **Invited speaker**, The 6th International Conference on Antibody Engineering, La Jolla, CA, USA, December 1995.

Title: Disulfide stabilized Fv fragments: Novel Approach in Antibody Engineering

3) **Plenary lecture**, International Conference on Immunotoxins, Myrtle Beach, SC, USA, June 1995.

Title: Recombinant Immunotoxins against Colon Cancer

4) The Yoram Avi-dor Lecture, Faculty of Biology, The Technion, November 1998.

Title: Recombinant Antibodies for Cancer Therapy and Diagnosis.

5) Guest Seminar, Department of Food Engineering and Biotechnology, Technion, May 1999.

Title: Protein Engineering of Recombinant Antibody Fragments for Cancer Therapy and Diagnosis.

6) **Invited speaker**, Symposium on Phage and Cell Display, The Laura Schwarz-Kipp Institute of Biotechnology, Tel Aviv University and the Israel Ministry of Science, June 1999.

Title: Recombinant antibodies with TCR-like specificity by phage-display.

7) Invited speaker, Biotechnology General Ltd. Rehovot, Israel, August 1999.

Title: Recombinant MHC-peptide complexes and their application.

8) **Invited speaker (plenary lecture)** The Danish Cancer Society Symposium, The Royal Danish Cancer Society, Copenhagen, Denmark, August 1999.

Title: Recombinant Immunotoxins for Targeted Cancer Therapy: Technology Closes In on Potential.

9) **Invited speaker**, Goldman Lichtman Mermorial Symposium in Oncology,

Cancer Vaccines and Immunotherapy, Hebrew University Medical School and Hadassah Medical Organization, Jerusalem, October 1999.

Title: Recombinant MHC molecules and their use for molecular analysis of cancer-specific immune response.

10) Guest Seminar, Faculty of Medicine, Ben-Gurion University at the Negev, Beer-Sheva, November 1999.

Title: Recombinant Antibodies by Phage Display: Isolation of Novel Targeting Molecules with Unique Specificities to Tumor Associated Antigens.

11) **Presentation**, The 29 Annual Meeting, The Israel Immunological Society, Hebrew University-Hadassah Medical School, Jerusalem, January 2000.

Title: Recombinant Antibodies with TCR-like specificity.

12) Guest Seminar, Unit for Research on Auto-immune diseases. Tel-Hashomer Medical Center and Sackler School of Medicine, Tel-Aviv University, March 2000.

Title: MHC tetramers for phenotypic characterization of tumor specific CTLs.

13) Guest Seminar, The Institute of Life Sciences and The Wolfson Centre for Applied Structural Biology, The Hebrew University of Jerusalem, Jerusalem, May 2000.

Title: Tumor Associated Antigens (TAAs) in Complex with Class I MHC Molecules: New Approaches for Targeted Cancer Therapy and For Studying Anti-Tumor Immune Responses.

14) Guest Seminar, Lautenberg Center For Tumor Immunology, Hebrew University Medical School, Jerusalem. January 2001.

Title: Tetrameric recombinant MHC/peptide complexes: A powerful tool to study anti-tumor immune responses and for the generation of recombinant antibodies with TCR-like specificity

15) **Invited speaker** (**Plenary lecture**), 30th meeting of the Israel Immunology Society, Rehovot, February 2001.

Title: MHC tetramers: a powerful tool in molecular immunology.

16) **Invited speaker**, Immunotherapy Program Seminars, Department of Dermatology, Charite, Humbold University, Berlin, Germany, April 2001.

Title: MHC tetramers and recombinant antibodies with TCR-like specificity for the

study of anti-tumor immune responses.

17) **Invited speaker**, Annual Scientific meeting of the Israel Association of Allergy and Clinical Immunology, Ramat-Gan, Israel, April 2001.

18) **Presentation**, 11th International Congress of Immunology, Stockholm, Sweden. July 2001.

Title: Critical role for CD8 in the binding of human cancer-specific MHC Tetramers to TCR.

19) Guest seminar, Department of Molecular Microbiology and Biotechnology, Tel Aviv University, October 2001

Title: Molecular engineering of cell specificity

20) Guest seminar, Faculty of life Sciences, Bar-Ilan University , November 2001

Title: Transforming T-cell specificity into high affinity antibody molecules

21) Guest seminar, Faculty of Medicine, Hebrew University, Departments of Biochemistry and Cancer Research, November 2001

Title: Engineering T-cell receptors.

22) **Invited speaker**, 3rd Meeting of the Israel Societies for Experimental Biology (FISEB), Eilat February 2002.

Title: Molecular Engineering of T-cell Receptors

23) Guest seminar, Division of Oncology, Sheba Medical Center, Tel-Aviv, May 2002.

Title: Recombinant antibodies with T-cell receptor-like specificity directed toward TAA.

24) **Invited Seminar,** Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda MD USA, August 2002,

Title: Recombinant TCR-like antibodies

25) **Plenary Speaker,** 32nd Annual Meeting of the Israel Immunology Society, Haifa,

Israel. February 2003.

Title: TCR-like antibodies

26) **Invited Speaker,** EU Workshop on "Cellular Transport Strategies for Targeting Epitopes, Drugs and Reporter Molecules" Budapest, March 2003

Title: Targeting tumor cells with recombinant T-Cell Receptor-like Antibodies and MHC-peptide complexes.

27) **Invited Plenary Speaker,** 2nd International Congress on recombinant Antibodies,

Munich, April 28-30, 2003

Title: Human recombinant antibodies with TCR-like specificity

28) **Invited Plenary Speaker,** 4th Annual CHI Recombinant Antibodies meeting, May 2003, Cambridge, MA. USA

Title: Human Recombinant Antibodies with TCR-like Specificity.

29) **Invited speaker,** Cancer Research UK Tumor Immunology Program, Institute of Molecular Medicine, University of Oxford, UK April 2002.

Title: TCR-like antibodies: new tools for the study of antigen presentation by tumor and viral-infected cells.

30) **Invited speaker,** Cancer Vaccines 2003, Cancer Research Institute International Symposium, September-October 2003 New York

Title: TCR-like recombinant antibodies: New tools to study antigen presentation And monitor vaccines.

31) **Invited Speaker,** 8th World Congress on Advances in Oncology and the 6th International Symposium on Molecular Medicine. October 2003, Greece.

Title: Recombinant MHC molecules and TCR-like antibodies.

32) **Invited Speaker,** XV. National Biophysics Congress, October 2003, Denizli,Turkey.

Title: From Membrane Biophysics into Cancer.

33) **Invited Speaker,** Fall Symposia on Vaccine Development, ISM-Israel Society for Microbiology, November 2003.

Title: Recruitment of CTL activity by MHC-antibody fusion proteins.

34) **Invited Plenary Speaker,** 2nd Multidisciplinary Colorectal Congress, February 2004, Noordwijk, The Netherlands

Title: Immunogenic epitopes as targets for immunotherapy in colorectal cancer.

35) **Invited Speaker**, 3rd National Biotechnology Week Meeting, Tel-Aviv, May 2004.

Title: Immunotherapy of Cancer

36) **Invited Speaker**, 12th International Congress of Immunology, Montreal, July 2004.

Title: Recombinant antibodies with peptide-specific, MHC-restricted specificity: Implications for study of antigen presentation and MHC-peptide structure.

37) **Invited chair**, Mini-symposium on Structre of MHC, 12th International Congress of Immunology, Montreal, July 2004.

Present Affiliation:

Faculty of Biology
Technion-Israel Institute of Technology
Technion City
Haifa 32000, Israel

Tel: (office) 972-4-8292785 ;
(lab) 972-4-8293592
Fax: 972-4-8225153
e-mail: reiter@tx.technion.ac.il
net: http://biology.technion.ac.il/reiter

Home: 20 Hasachlav st.

Haifa 34790, Israel Tel: 972-4-8265719

Current Research Activities:

Molecular immunology, Cancer immunology, Antibody engineering, Recombinant antibodies, MHC-peptide complex, Cellular Immunology, Antibody phage display

1. Recombinant antibody fragments for cancer therapy and diagnosis.
2. Antibody phage display libraries for the isolation of novel reagents for targeting cancer and autoimmune disorders.
3. Intracellular expression of recombinant antibodies and targeting to subcellular compartments.
4. Protein engineering of recombinant antibody and T-cell receptor Fv fragments.
5. Study of antigen presentation in cancer and viral diseases
6. Recombinant MHC molecules.
7. Targeting MHC to tumor cells.

Students and Research staff

Liat Binyamin Ph.D student (11/1999-)

Malki Epel Ph.D student (10/2000-)

Roy Noy Ph.D student (4/2002-)

Maya Hauz Ph.D student (10/2002-)

Einav Kalchevsky Ph.D student (10/2001-)

Hila Novak PhD student (direct path) (10/2002-)

Orian Mekler M.Sc student (10/2002-)

Efrat Zahavi M.Sc student (10/2002-)

Kfir Oved Ph.D student (direct path) (3/2003-)

Arbel Artzi M.Sc student (3/2004-) with Prof Uri Sivan-Faculty of Physics

Yael Michaeli MSc student (10/2004-)

Inbal Zafir MSc student (10/2004-)

Keren Gueta MSc student (10/2004-)

Ron Piran PhD student (10/2003-) With Prof Ehud Keinan -Faculty of Chemistry

Tali Lev,Ph.D, Post Doctoral fellow (9/2000-7/2003)

Galit Denkberg, Ph.D Post Doctoral Fellow (1/2003-)

Dina Segal, M.Sc, Research associate (10/1998-)

David Hazan, MD, clinical research (1/2000-1/2001)

Graduated students

Revital Niv M.Sc (2/1999-2/2001)

Maya Hauz M.Sc (10/2000-10/2002)

Galit Denkberg, Ph.D (12/1998-1/2003)

Cyril Cohen Ph.D (10/1999-10/2003)

Grants and Patents:

Patents:

1. **US 09/297,851; David FitzGerald,** Yoram Reiter, **Ira Pastan : Protease-activated**
Pseudomonas Exotoxin A-like proprotein.

2. US 09/534,966 **Yoram Reiter**, Galit Denkberg, Cyril Cohen : SINGLE CHAIN CLASS I MAJOR HISTO-COMPATIBILITY COMPLEXES, CONSTRUCTS ENCODING SAME AND METHODS OF GENERATING SAME.

3. PCT/ IL01/00260, **Yoram Reiter**, Galit Denkberg, Cyril Cohen : SINGLE CHAIN CLASS I MAJOR HISTO-COMPATIBILITY COMPLEXES, CONSTRUCT ENCODING SAME AND METHODS OF GENERATING

SAME.

4. US10/073,301; **Yoarm Reiter**, Galit Denkberg : ANTIBODY HAVING A T-CELL RECEPTOR-LIKE SPECIFICITY, YET HIGHER AFFINITY, AND THE
USE OF,SAME IN THE DETECTION AND TREATMENT OF CANCER, VIRAL INFECTION AND AUTOIMMUNE DISEASE.

5. US 09/065,329; **Yoram Reiter**, Hennie Hoogenboom, Dyax coorperation : MHC-
PEPTIDE COMPLE BINDING LIGANDS.

6. US 60/298,915; **Yoram Reiter**, Avital Lev : METHODS AND PHARMACEUTICAL COMPOSITIONS FOR IMMUNE DECEPTION, PARTICULARLY USEFUL IN THE TREATMENT OF CANCER.

7. US 10/654,037 Uri Sivan and **Yoram Reiter**
Electronic Receptor and Its Application to Controlling Biological Processes

RESEARCH GRANTS

1. 1998-1999, Research grant from the "Alon" fellowship; Israel Council for Higher Education, (VATAT) Ministry of Education; 29,000$.

2. 1999, The L. Naftali Science Foundation for Biology and Medicine. Award 12,250$.
Study of antigen presentation by cancer cells (Project 140-611).

3. 1999-2002, TEVA Award for young scientists in Life-Science and Medicine, TEVA Pharmaceuticals Ltd; 30,000 NIS.
   Novel approaches for the treatment of autoimmune diseases: Recombinant Antibodies with T-cell Receptor-like specificity (Project 140-619).

4. 1999, Research grant, Taub Foundation for Biological Research; 10,000$
   Recombinant antibodies for cancer therapy and diagnosis (Project 140-598).

5. 1999-2004, Research Career Development Award, ICRF-Israel Cancer Research Fund (USA) (5 year award); 150,000$.
   Study of antigen presentation by tumor cells using recombinant antibodies (Project 140-594)

6. 1999-2001, Research grant, Peptor Ltd., Rehovot, Israel (as part of the MAGNET Program, Consortium "DAAT", Ministry of Industry and Trade); 180,000$.
   Recombinant antibodies against class I MHC-peptide complexes (Project 140-598).

7. 1999-2002, Research grant, The Israel Science Foundation (ISF), The Israel Academy of Sciences and Humanities; 158,577$.
   Study of antigen presentation by tumor cells and development of novel targeting Reagents by recombinant antibodies with T-cell receptor-like specificity (Project 140-588).

8. 1999, Grant for research equipment, The Israel Science Foundation, The Israel Academy of Sciences and Humanities 19,000$ (Project 140-588).

9. 1999, Grant for infrastructure equipment for a new faculty member (Genetic analyzer DNA sequencing system), The Israel Science Foundation (ISF), The Israel Academy of Sciences and Humanities; 30,000$.

10. 1999-2000, Research Award, YAEL Foundation, Biosense Ltd. Israel, 60,000$.

    The tumor cell as an antigen-presenting cell: analysis of cancer-related peptides presented by class I MHC and development of novel targeting agents by combinatorial antibody phage-display technology (Project 140-621).

11. 2000-2001, Research grant, Israel Cancer Association, Israel; 120,000 NIS.

    Analysis of antigen presentation by melanoma cells and anti-tumor immune responses using multimeric recombinant class I MHC/peptide complexes (Project 140-641)

    A collaboration with Dr. David Hazzan and Prof. Eitan Shiloni - Carmel Medical Center, Haifa.

12. 2000, TEVA Research Grant Award, Teva Pharmaceutical Industries Ltd; 65,000 NIS.

    Rapid isolation of human antibodies from phage display libraries (Project 140-651).

13. 2001-2003, Research Grant, Chief Scientist, Ministry of Health; 180,000 NIS

    Study of antigen presentation with recombinant TCR-like antibodies (project 140-654)

14. 2001-2002, Research Grant, Medis-El advanced technologies Ltd.;115,000$,

    Using scMHC tetramers and the CellScan to develop new early cancer diagnosis (project 140-690)

15. 2002-2003, Research Grant, DFG-Deutsche Forschungsgemeinschaft , Germany,

    64,933 Euro,

    Study of antigen presentation in melanoma using recombinant antibodies with TCR-specificity (project 140-672)

16. 2002-2004, Research Grant, Teva Pharmaceuticals Ltd and Magnet program, Ministry of Trade and Industry (MAGNETON project), 627,000 $ (2002/3 1st year 257,000 $; budget for 2nd year 2003/4 370,000$) + 240,000 $ for equipment (FACS cell sorter + FPLC system + Flter beta counter)

Antibody-mediated targeting of MHC-peptide complexes to tumor cells (project 140-702)

17. 2002-2003, Research Grant, "NOFAR" project, Magnet program, Ministry of Trade and Industry and Teva Pharmaceuticals; 100,000 $

Antibody-based inhibitors for MDR (project 140-721)

18. 2002-2005, Research grant, The Israel Science Foundation (ISF), The Israel Academy of Sciences and Humanities; 201,000 $.

Study of antigen presentation by tumor cells using T-cell receptor-like antibodies (Project 140-706).

19. 2004, Teva Pharmaceuticals Ltd., 415,000 $

Antibody-mediated targeting of MHC-peptide complexes to tumor cells (project 2003399)

20. 2004-2007, Israel Science Foundation, Bikura Program; 102,600 $ (with Prof. Uri

Sivan, Physics)

Talking to bio-molecules in their language.

21. 2005, Teva Pharmaceuticals LTd., 479,801 $

Antibody-mediated targeting of MHC-peptide complexes to tumor cells

Total grants since 1998: 3,190,000 $

Current grant applications:

1) NIH/Gates Grand Challenge grant application (EU consortium)
2) NIH RO1 grant application

PUBLIC PROFESSIONAL ACTIVITIES:

1999, 2001, 2002
Member of the Scientific Advisory Board for Grants in Cancer Research, Ministry of Health, Jerusalem, Israel.

1999-2001
Member of Scientific Advisory Board, Immunology Section, ISF-Israel Science Foundation, Israel Academy of Sciences and Humanities, Jerusalem, Israel.

2000-
Member of Scientific Advisory Board on Cancer Immunotherapy, Hadassah Medical Center, Sharet Institute for Oncology, Hebrew University of Jerusalem, Jerusalem, Israel.

2002-2003
Organizing committee and Scientific committee, 32nd Annual Meeting of the Israel Immunology Society.

2003
Chairman, Advisory Board for Grants in Cancer Research, Ministry of Health, Jerusalem, Israel

2003-
Scientific advisory board (SAB), Viventia Biotech. Ltd , Toronto, Canada

2004-

Scientific advisory board (SAB), The Ella Institute for Treatment and Research of Melanoma, Sheba Medical Center.

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

1995- American association for Cancer Research.

1998- American association of immunologists.

1999- Israel Immunology Society

BIBLIOGRAPHY

1. **Reiter, Y.**, and Fishelson, Z.: Tumor cell lysis by Antibody-complement conjugates. *Complement* 4:154, 1987.

2. Fishelson, Z., and **Reiter, Y.**: Antibody-C3b conjugates: a novel reagent for immunotherapy. *J. Cell. Biochem. Suppl* 12E: 176,1988.

3. Fishelson, Z., Kopf, E., and **Reiter, Y.**: Phosphorylation, phophatidylinositol turnover and resistance to complement damage. *FASEB J.* 2: A872, 1988.

4. **Reiter, Y.**, and Fishelson, Z.: Targeting of complement to tumor cells by heteroconjugates composed of antibodies and of the complement C3b. *J. Immunol.* 142: 2771, 1989.

5. **Reiter, Y.**, and Fishelson, Z.: Killing of human tumor cells by antibody-C3b conjugates and human complement. In Radwell, J.D. (Ed.): Targeted Antibodies for Diagnosis and Therapy. New York, Marcel Dekker Inc., 1989, pp. 119-1135.

6. Fishelson, Z., and **Reiter, Y.**,: Monoclonal Antibody-C3b conjugates: killing of K562 cells and selection of a stable complement resistant variant. In

Grogoman, J., Evans, C., and Golde, D. (Eds.):Mechanisms of Action and Therapeutic Application on Biologicals in Cancer and Immune Deficiency Disorders. New York, Alan R. Liss, Inc., 1989, pp. 272-282.

7. Fishelson, Z., Kopf, E., Pass, Y., Ross, L., and **Reiter, Y.**: Protein phosphorylation as a mechnism of resistance against complement damage. *Prog. Immunol.* 7: 205, 1989.

8. Yefenof, E., Benizri, R., **Reiter, Y.**, Klein, E., and Fishelson, Z.: Potentiation of target cell sensitivity to NK lysis by antibody-C3b/iC3b heteroconjugates. *J. Immunol.* 144: 1538, 1990.

9. **Reiter, Y.**, and fishelson, Z.: De novo protein synthesis is induced in human leukemic cells by antibody and complement *FASEB J.* 4: A1872, 1990.

10. Fishelson, Z., and **Reiter, Y.**: sublytic complement attack potentiates the resistance of tumor cells to lytic doses of complement. *Compl Infl.* 8: 150, 1991.

11. Reiter, Z., **Reiter, Y.**, Fishelson, Z., Loyter, A., Nussbaum, O., and Rubinstein, M.: ClassI MHC antigens are not associated with resistance to NK cell mediated cytotoxicity. *Immunobiol.* 183: 23, 1991.

12. **Reiter, Y.**, and Fishelson, Z.: Complement membrane attack complexes induces synthesis of large complement induced proteins (L-CIP) in human leukemic cells. *Mol. Immunol.* 29: 771, 1992.

13. **Reiter, Y.**, Cibotariu, A., and Fishelson, Z.: Sublytic complement attack protects tumor cells from lytic doses of antibody and complement. *J. Immunol.* 22: 1207, 1992.

14. Brinkmann, U., **Reiter, Y.**, Jung, S.-h., Lee, B., and Pastan, I.: A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl.*

Acad. Sci. USA 90: 7538, 1993.

15. **Reiter, Y.**, Brinkmann, U., Webber, K.O., Jung, S.-h., Lee, B.K., and Pastan, I.: Engineering interchain disulfide bonds into conserved framework regions of Fv fragment: improved biochemical characteristics of recombinant immunotoxins containing disulfide-stablilized Fv. *Protein Eng.* 7: 697, 1994.

16. **Reiter, Y.**, Brinkmann, U., Kreitman, R.J., Jung, S.-h., Lee, B.K., and Pastan, I.: Stabilization of the Fv fragments in recombinant immunotoxins by disulfide-stabilized Fv fragment. *Biochemistry* 33: 5451, 1994.

17. **Reiter, Y.**, Pai, L., Brinkmann, U., and Pastan, I.: Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Cancer Res.* 544: 2714, 1994.

18. **Reiter, Y.**, Kreitman, R.J., Brinkmann, U., and Pastan, I.: Cytotoxic and antitumor activity of a recombinant immunotoxin composed of disulfide-stabilized anti Tac(Fv) fragment and a truncated *Pseudomonas* exotoxin. *Int. J. Cancer.* 58: 142, 1994.

19. **Reiter, Y.**, Brinkmann, U., Jung, S-h, Lee, K., Kasprzyk, P.G., King, C.R., and Pastan, I.: Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide-stabilization of the Fv fragment. *J. Biol. Chem.* 269: 18327, 1994.

20. **Reiter, Y.**, Kurucz, I., Brinkmann, U., jung, S.h., Lee, B., Segal, D.M., and Pastan, I.: Construction of a disulfide stabilized TCR Fv fragment indicates that antibody and TCR Fv frameworks are very similar in structure. *Immunity* 2: 281, 1995.

21. Webber, K.O., **Reiter, Y.**, Brinkmann, U., Kreitman, R.J., and Pastan, I.: Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-

Tac antibody: comparison with its single-chain analog. *Mol. Immunol.* 32: 249, 1995.

22. Benhar, I., **Reiter, Y.**, Pai,.L., and Pastan,I.: Administration of disulfide-stabilized B1(dsFv)-PE38 and B3(dsFv)-PE38 by continuos infusion increases their efficacy in curing human tumor xenografts in mice. *Int. J. Cancer.* 62: 351, 1995.

23. **Reiter, Y.**, Ciobotariu, A., Jones, J., Morgan, B.P., and Fishelson, Z.: Complement membrane attack complex, perforin, and bacterial exotoxin induce in K562 cells calcium-dependent cross-protection from lysis. *J. Immunol.* 155: 2203, 1995.

24. **Reiter, Y.**, Brinkmann, U., Jung, S-h., Lee. B., and Pastan, I.: Disulfide-stabilization of antibody Fvs: computer prediction and experimental evaluation. *Protein Eng.* 8: 1323, 1995.

25. Fishelson, Z., Jones, J., and **Reiter, Y.**, : Immunologic tachyphylaxis, a mechanism of cellular protection from complement, perforin and bacterial exotoxins. *FASEB J.* 10: 2015, 1996.

26. **Reiter, Y.**, and Pastan, I.: Antibody engineering of recombinant Fv-immunotoxins for improved targeting of cancer. *Clin. Cancer Res.* 2: 245, 1996.

27. **Reiter, Y.**, Wright, A.F., and Pastan, I.: Recombinant single-chain and disulfide-stabilized Fv immunotoxins that cause complete regression of a human colon cancer xenograft in nude mice. *Int. J. Cancer.* 67: 113, 1996.

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Antibody-mediated targeting of human single-chain class I MHC with covalently linked peptides induces efficient killing of tumor cells by tumor or viral-specific cytotoxic T lymphocytes

Kfir Oved*, Avital Lev*, Roy Noy, Dina Segal, and Yoram Reiter

Faculty of Biology, Technion-Israel Institute of Technology
Haifa 32000, Israel

* equal contribution

Running Title: scFv-MHC fusion protein with covalently linked peptide

Address correspondence to: Dr. Yoram Reiter, Department of Biology, The Technion-Israel Institute of Technology, Technion City, Room 333, Haifa 32000, Israel. Tel: 972-4-8292785; Fax: 972-4-8225153; E-mail: reiter@tx.technion.ac.il

ABSTRACT

Soluble forms of human MHC class I HLA-A2 were produced in which the peptide binding groove was uniformly occupied by a single tumor or viral-derived peptides attached via a covalent flexible peptide linker to the N terminus of a single-chain β-2-microglobulin-HLA-A2 heavy chain fusion protein. A tetravalent version of this molecule with various peptides was found to be functional. It could stimulate T cells specifically as well as bind them with high avidity. The covalently linked single chain peptide-HLA-A2 construct was next fused at its C-terminal end to a scFv antibody fragment derived from the variable domains of an anti-IL-2R α subunit-specific humanized antibody, anti-Tac.

The scFv-MHC fusion was thus encoded by a single gene and produced in *E. coli* as a single polypeptide chain. Binding studies revealed its ability to decorate Ag-positive human tumor cells with covalent peptide single-chain HLA-A2 (scHLA-A2) molecules in a manner that was entirely dependent upon the specificity of the targeting Antibody fragment. Most importantly, the covalent scHLA-A2 molecule, when bound to the target tumor cells, could induce efficient and specific HLA-A2-restricted, peptide-specific CTL-mediated lysis. These results demonstrate the ability to generate soluble, stable, and functional single-chain HLA-A2 molecules with covalently linked peptides, which when fused to targeting antibodies, potentiate CTL killing. This new approach may open the way for the development of new immunotherapeutic strategies based on antibody targeting of natural cognate MHC ligands and CTL-based cytotoxic mechanisms.

INTRODUCTION

Cell-mediated cytotoxicity via anti-tumor $CD8^+$ T lymphocytes is one of the major means used by the immune system to destroy transformed cells. According current immune surveillance theory, the immune system is continuously locating and destroying transformed cells, but some of them succeed in escaping from effective immune response and consequently become tumors (1-5).

Tumor evasion from immune response is a well-established phenomenon demonstrated in numerous studies; it is caused by a wide variety of suggested mechanisms (1-5). Among these mechanisms are: the production of suppressive cytokines, loss of immunodominant peptides, resistance to killing mechanisms (apoptosis), loss of MHC class I, and a few other additional mechanisms (1-5). One of the major evasion mechanisms, which has been shown to be strongly correlated with tumor progression, is the loss/down-regulation of MHC class I molecules. This evasion mechanism is abundant in many tumor lesions and can result from a number of different mutations. Several studies revealed some weak spots in the MHC class I loading and presentation route including loss of beta-2 microglobulin, TAP1/TAP2 mutations, LMP mutations, loss of heterozygocity in the MHC genes, and down-regulation of specific MHC alleles.

Two major strategies are currently being used to increase the antitumor effectiveness of the immune system's cellular arm: (1) active immunization of patients with peptides known to be recognized by T lymphocytes and activate them and (2) adoptive transfer therapies that enable the selection, activation, and expansion of highly reactive T-cell subpopulations with improved anti-tumor potency. In the first approach, MHC-restricted

peptides derived from recently identified tumor-associated antigens (such as gp100, the MAGE group, NY-ESO-1, and many others) are used. These antigens are highly specific due to their exclusive expression in specific tissues (6-10).

The second strategy, adoptive transfer, has recently shown impressive results in metastatic melanoma patients where highly selected tumor-reactive T cells against different overexpressed self-derived differentiation antigens were used (11-13). In this approach a persistent clonal repopulation of T cells, proliferation *in vivo*, functional activity, and trafficking to tumor sites were demonstrated.

Another main approach in immunotherapy is passive immunization which utilizes the humural arm of the immune system. In this approach high-affinity monoclonal antibodies (mAbs) directed against cell surface tumor-associated antigens are systemically injected. Notably, it has demonstrated statistically significant anti-tumor activities in clinical trials, for example, with B-cell lymphoma and breast carcinoma (14, 15).

We have recently presented a new immunotherapeutic approach that takes advantage of two well-established areas: (1) the known effectiveness of $CD8^+$ cytotoxic T lymphocytes in the elimination of cells presenting highly immunogenic MHC/peptide complexes, and (2) the tumor-specific-cell surface antigens targeting via recombinant fragments of antibodies (mainly scFvs) (16). This approach utilizes a recombinant fusion protein composed of two functionally distinct entities: (i) a single-chain MHC class I molecule that carries a highly immunogenic tumor or viral-derived peptide, and (ii) a tumor-specific, high-affinity scFv fragment. Several groups have previously shown that a biotinylated MHC/peptide multimerized on streptavidin or monomeric HLA-A2/influenza (Flu) matrix peptide complexes coupled via chemical conjugation to tumor-

specific antibodies could induce *in vitro* T-lymphocyte-mediated lysis of coated tumor cells (17-21). However, these approaches utilized chemical conjugation and used whole antibodies or larger fragments (Fab), which limit their production and homogeneity owing to the coupling strategy as well as their limited tumor penetration capabilities due to their large size. In our approach, a genetic fusion is created between a single-chain recombinant HLA-A2 and tumor specific scFvs. These fusions were shown to be functional *in vitro* and *in vivo*, thus, they were able to specifically induce T-lymphocyte-mediated *in vitro* and *in vivo* lysis of target-coated tumor cells (22).

However, one potential problem with MHC-peptide constructs is the stability of the antigenic peptide in the MHC peptide binding groove because the peptide can dissociate from the complex. In this work we addressed this problem and took the molecular structure of these fusions one step further and created them with a covalently bonded antigenic peptide. We hypothesize that the covalent attachment of the peptide will make the complex more stable by its ability to continuously and efficiently rebind the covalently attached peptide.

Soluble MHC molecules occupied uniformly by single peptides are very useful in both structural and biological studies of MHC function. Several expression systems have been developed to produce soluble MHC class I or class II molecules without a stably bound peptide that, under the appropriate conditions, can subsequently be loaded with single peptides, sometimes requiring the refolding of denatured MHC molecules in the presence of the peptide, which is also performed in making scHLA-A2 complexes and their fusion to the scFv. Alternatively, strategies for covalently attaching an antigenic peptide to the N terminus of the MHC were developed for murine class I and class II MHC. These

molecules have been expressed in both soluble and membrane associated forms (23-31). However, using such class I molecules with human MHC was not previously reported.

In this study we have constructed and produced a single-chain HLA-A2 molecule with tumor and viral-derived, covalently bound antigenic peptides. First, we will demonstrate that these constructs are functional; as tetramers they can stain T cells and activate them.

Next, after fusing these covalent peptide scHLA-A2 constructs to a tumor targeting scFv antibody fragment, we will demonstrate that the new fusion molecule can maintain its dual activity: bind tumor target cells through the scFv moiety as well as mediate potent and effective cytotoxicity through the recruitment of CD8+ T cells whose specificity is governed by the covalently linked HLA-A2-restricted and occupied single antigenic peptide. Using this approach, we have produced functional human MHC molecules that are highly stable and are fully occupied by a single peptide. Importantly, we have demonstrated the ability to fuse these constructs to targeting antibodies and generate fusion molecules for immunotherapeutic approaches that may bridge antibody and T lymphocyte attack on cancer cells.

Results

Construction and production of a single-chain HLA-A2 with covalently linked peptides

We previously reported on the construction of a single-chain HLA-A2 in which the β_2M gene was fused through a flexible peptide linker to the three extracellular domains (α1,α2,and α3) of the HLA-A2 heavy chain gene (Figure 1A). This construct was analyzed in detail for its biochemical and biological properties and was found to be functional (34,36). To construct a single-chain HLA-A2 (scHLA-A2) with a covalently linked peptide, the HLA-A2-restricted peptides, G9-209M, G9-280V, $MART_{27-35}$, derived from melanoma differentiation antigens gp100 and MART1 (37-39) and the EBV-derived peptide $GLC_{280-288}$ (40,41) were genetically fused to the engineered scHLA-A2 (Fig.1B) by overlap extension PCR. This construct was termed covalent-single-chain HLA-A2 (cov-scHLA-A2). In this construct, the antigenic peptide was fused to the N-terminus of the human β_2M gene through a 15-aa long flexible linker ((Gly4-Ser)$_3$). The length of the linker was selected by molecular modeling to ensure correct folding of the molecule. In both the scHLA-A2 (Fig. 1A) and the cov-scHLA-A2 (Fig. 1B) constructs a Biotin ligase sequence tag for site-specific biotinylation was engineered at the C-terminus of the HLA-A2 gene for the generation of MHC tetramers. First, we produced the cov-scHLA-A2 to assess its correct folding and functionality and to compare its properties with the previously characterized scHLA-A2. Once this was performed, described next, we fused the covalent scHLA-A2 construct to a scFv antibody fragment (Fig. 1C) and demonstrated that this construct could mediate antibody-mediated peptide-specific CTL killing of target cells that were coated with the newly engineered

cov-scHLA-A2 molecule. The cov-scHLA-A2 molecules were expressed in *E. coli* BL21 cells and they accumulated as intracellular inclusion bodies. As shown in Fig 2A, SDS-PAGE analysis of the isolated inclusion bodies revealed that they contained more than 90% of the recombinant protein, and no major differences in the expression level were observed when the 4 HLA-A2-restricetd peptides were fused to the scHLA-A2 gene. The isolated inclusion bodies were solubilized, reduced, and refolded *in vitro* in a redox-shuffling buffer system. Monomeric soluble cov-scHLA-A2 complexes were purified from the refolding solution by ion-exchange chromatography on Q-Sheparose. As shown in Figure 2B, SDS-PAGE analysis revealed a highly purified monomeric complex with the expected molecular weight of 48 Kd. For controls we produced scHLA-A2 complexes with the 4 HLA-A2-restricted peptides by *in vitro* refolding of inclusion bodies with a 10-fold molar excess of the antigenic peptide in the refolding solution (34, 36).

Characterization and biological function of the covalent peptide scHLA-A2 complexes

To first assay the folding of the cov-scHLA-A2 complexes, we immobilized them onto BSA-biotin-coated immuno plates that were incubated with streptavidin. Biotinylated cov-scHLA-A2 complexes were subsequently immobilized so that the BSA-biotin-Streptavidin bridge enabled their efficient immobilization without affecting their folding.

The folding of the complexes was tested by binding the conformational MAb W6/32, which recognizes HLA complexes only when folded correctly and when containing peptide. As shown in Figure 3A, covalent scHLA-A2 complexes made by fusion of the 209, 280, and EBV peptides reacted with W6/32, and the binding intensity was

indistinguishable from the reactivity of control complexes generated with a non-covalent bound peptide (scHLA-A2). These testes were performed at saturating concentrations of the MHC-peptide complex. In addition, titration experiments comparing the reactivity of the covalent and non-covalent complexes at increasing concentrations also did not revealed any difference in reactivity with MAb W6/32 (data not shown). The cov-scHLA-A2 complex with the MART1 peptide exhibited reduced reactivity with W6/32 (Fig. 3A) compared with the refolded scHLA-A2/MART1 complex.

These results demonstrate that three out of the four cov-scHLA-A2 complexes that were generated are correctly folded, as judged by their reactivity with W6/32. The poor reactivity of the cov-scHLA-A2/MART complex may result from instability or improper folding.

Next, we tested the biological function of the cov-scHLA-A2 complexes using two biological assays that test their reactivity with their cognate TCR, namely by T cell re-activation and staining by flow cytometry. We used the cov-scHLA-A2 complexes, containing the four peptides, to generate tetramers by site-specific biotinylation through the BirA tag at the C-terminus of the construct. As shown in Figure 3B, the cov scHLA-A2 tetramers generated with the EBV peptide induced appreciable specific T-cell re-activation (because activated established CTLs are being used) of an EBV peptide-specific CTL line, as measured by the release of IFN-γ into the culture supernatant. The level of IFN-γ release was similar when T cells were incubated with the cov-scHLA-A2/EBV or scHLA-A2/EBV tetramers (Fig. 3B). In contrast, control cov-scHLA-A2 or scHLA-A2 containing the melanoma gp100-derived peptides 209 and 280 did not induce activation. Similar results were observed for cov-scHLA-A2 tetramers generated with

covalently linked 209 or 280 peptide; thus, they were able to specifically activate the 209 and 280-specific T-cell clones, respectively (data not shown). Next, we tested the staining of peptide-specific T cells by these cov-scHLA-A2 tetramers. As shown in Figure 4, the cov-scHLA-A2 complexes generated with EBV (Fig. 4A) and gp100-derived peptides 209 (Fig. 4B) and 280 (Fig. 4C) exhibited excellent, high avidity and specific staining intensities of peptide-specific CD8+ T cells that were indistinguishable from the staining observed when non-covalent scHL-A2 complexes were used. Controls were corresponding CD8+ T cells that were stained with a cov-scHLA-A2 complex containing a different covalently linked peptide. The data presented in Figure 3B (IFNγ release) and Figure 4 (tetramer staining) indicate that the cov-scHLA-A2 complexes are biologically functional. They can activate the corresponding T cells and can stain them; thus, they maintain the specific binding capabilities to the TCR of a native class I MHC and as shown herein, their reactivity and functioning are as efficient as the scHLA-A2 complexes folded around the antigenic peptide (non covalently linked).

Generation and characterization of a cov-scHLA-A2 complex fused to a targeting scFv antibody fragment

To test that our scFv-mediated MHC targeting approach can function with a scHLA-A2 construct that contains a covalently linked peptide, we genetically fused to the C-terminus of the cov-scHLA-A2 gene a scFv antibody fragment gene derived from anti-Tac, a monoclonal antibody recognizing CD25, the α subunit of the IL-2 receptor (*Tac*) (Fig. 1C) (32). We have generated three such constructs with covalently linked gp100-derived peptides 209 and 280 as well as the EBV-derived peptide. As a control we used

the scHLA-A2 construct that is genetically fused to the anti-Tac scFv and folded around the 209, 280, and EBV peptides (Fig. 1D). To produce the cov-scHLA-A2/αTac(scFv) fusion proteins, we subcloned the relevant fusion gene into T7 promotor-based expression vectors for expression in *E. coli* BL21 cells. Following induction with IPTG, large amounts of recombinant protein accumulated as intracellular inclusion bodies. SDS-PAGE analysis of isolated and purified inclusion bodies revealed that the recombinant fusion protein, with the correct size, constituted 80-90% of the total protein isolated from inclusion bodies (not shown). The inclusion bodies were isolated, solubilized, reduced, and refolded *in vitro* in a renaturation buffer system, which contained redox-shuffling and aggregation-preventing additives. The cov-scHLA-A2/αTac(scFv) fusion molecules were purified from the refolding solution by ion-exchange chromatography using Q-Sepharose columns. As shown in Fig. 2C, non-reducing SDS-PAGE analysis of peak fractions revealed the presence of monomeric cov-scHLA-A2/αTac(scFv) molecules having the correct molecular weight of ~67 KDa.

Proper folding and binding of the purified fusion molecules were tested by evaluating the ability of the cov-scHLA-A2/scFv to bind its target antigen, CD25. Recombinant purified CD25 protein was immobilized to immunoplates and the binding of the fusion molecule was tested. Binding was monitored by using the conformation-sensitive MAb W6/32. As shown in Fig.5A, the cov-scHLA-A2/αTac(scFv) molecules generated with three covalently linked peptides, 209, 280, and EBV bound in a dose-dependent manner to CD25, suggesting that the two different functional domains of the molecule, the covalently linked scHLA-A2-peptide complex effector domain and the antibody scFv targeting domain are properly folded and functional. This was demonstrated by the ability

of the scFv element to bind CD25, on the one hand, and to recognize the cov-scHLA-A2-peptide complex by the conformation-sensitive mAb, on the other. We also compared the binding to immobilized CD25 of the cov-scHLA-A2/scFv with non-covalent scHLA-A2 folded around the 209 peptide. As shown in Figure 5B and C, the binding intensities of w6/32 to the covalent scHLA-A2 fusion were much higher than the non-covalent scHLA-A2 folded with the 209 peptide. Since w6/32 is a conformation-sensitive antibody that is used to assess HLA-peptide complex stability, these data may suggest that the cov-scHLA-A2/scFv fusion is more stable than the non-covalent scHLA-A2/scFv fusion.

Using flow cytometry, we examined the ability of the cov-scHLA-A2/scFv fusion molecules to bind to tumor cells expressing CD25.

As a model, we used target cells that are HLA-A2-negative, so that the reactivity of the anti-HLA-A2 mAb will indicate the binding of the cov-scHLA-A2/scFv fusion molecule to the cell surface. These HLA-A2-negative target cells simulate the extreme case in which a target tumor cell loses its HLA-A2 expression, thus rendering it unsusceptible to HLA-A2-restricted CTL killing. We first used A431 human epidermoid carcinoma cells that were stably transfected with the CD25 gene (ATAC4 cells) (33), and compared the staining of transfected versus non-transfected cells. The binding of the cov-scHLA-A2/aTac-scFv molecule to the cells was monitored using an anti-HLA-A2 mAb BB7.2 as a primary antibody, followed by a FITC-labeled secondary antibody. The antibody BB7.2 and not W6/32 is now being used because it is specific for HLA-A2 while W6/32 reacts with all HLA alleles and thus cannot be used to detect only HLA-A2 on the cell surface of cells. The expression of the CD25 target antigen was monitored with anti-Tac MAb from which the scFv fragment was derived. As shown in Fig. 6A, A431 parental cells do

not express CD25; however, the CD25-transfected ATAC4 cells express high levels of the antigen (Fig. 6B). Neither cell line was HLA-A2-positive (Fig. 6D, G). However, when ATAC4 but not A431 cells were preincubated with the cov-scHLA-A2/aTac(scFv) molecule containing either covalently linked 209 or 280 gp100-derived peptides, they produced positive anti-HLA-A2 staining, indicating that they were coated with HLA-A2-peptide complexes via the scFv antibody fragment (Fig. 6D,G and Fig. 7H,K, respectively). The staining was dose-dependent (Fig 6F,J) and when the binding of the cov-scHLA-A2/scFv fusion protein with the covalent bound peptide was compared to the binding of the non-covalent scHLA-A2/scFv molecule, a moderate but reproducible increase in reactivity was observed for the 280-derived covalently linked molecule (Fig. 6H). Next, we tested the binding of cov-scHLA-A2/aTac(scFv) to HUT102W leukemic cells, which as shown by the binding of anti-Tac and BB7.2 mAb, respectively, express CD25 (Fig. 6C) but lack HLA-A2 expression (Fig. 6E, I). As shown in Fig. 6E and 6I, the ATL leukemic HUT102W cells expressing CD25 were positively stained for HLA-A2 only when preincubated with the cov-scHLA-A2/aTac(scFv) molecule. This binding was dose- dependent and saturation of binding was achieved in nanomolar range concentrations of the molecule (data not shown). Similar results were observed with CD25-positive ATL, HLA-A2-negative CRII cells (data not shown). These results demonstrate that the cov-scHLA-A2/aTac(scFv) molecule can bind its antigen, as displayed in the native form on the cell surface. Estimation of the percentage of fully functional molecules having both scFv binding and stable MHC peptide is estimated according to the assays presented herein to be in the range of 15-25%. This is quantified

by the scFv ability to bind to the target antigen and the reactivity of the MHC-peptide portion of the molecule with the conformation-dependent antibody W6/32.

Biological activity of the cov-scHLA-A2/scFv fusion molecule

To test the ability of the cov-scHLA-A2/scFv molecule to potentiate the susceptibility of HLA-A2- negative cells to CTL-mediated (HLA-A2-dependent) killing, we performed cytotoxicity assays on CD25-transfected ATAC4 cells. First, we incubated radiolabeled target cells with cov-scHLA-A2/aTac(scFv) fusion molecules generated with covalently linked gp100-derived peptides 209 and 280 or an EBV-derived peptide, and then we tested them in a ^{35}S-methionine-release assay in the presence of HLA-A2-restricted melanoma gp100- or EBV-peptide-specific CTLs. To first determine the killing potential of the gp100-specific CTLs we performed cytotoxicity experiments on FM3D melanoma cells which express high levels of gp100 and HLA-A2 (data not shown) As shown in Figure 7A, these CTLs were able to kill 35-40% of target cells at an E:T ratio of 20:1 and above. See assays were served as a comparison curve to the activity induced by these CTLs in the presence of the HLA-A2/scFv fusions and thus results are presented as relative cytotoxicity to the assay in Fig. 7A. In addition in preliminary studies ensured that the scHLA-A2/scFv molecules employing anti-Tac will not have any effect on CTLs because they express the target antigen CD25. To avoid any activation effects the target cells are being incubated first with the fusion protein, washed, and only after are incubated with CTLs. In addition, the affinity of the anti-Tac antibody is very high (1nM) and thus its off rate is very slow so that it dissociation from target cells and re-binding to the CTLs is unlikely to have a profound effect on CTLs during the incubation with the

fusion molecule-coated target cells. As shown in Figure 7B,D,E,F cov-scHLA-A2/aTac(scFv) effectively induced CTL-mediated lysis of CD25-positive HLA-A2-negative ATAC4 (Fig. 7B) and HUT102W (Fig. 7E) cells. When these cells were incubated with CTLs alone, without prior coating with the fusion protein, no significant cytotoxicity was observed and A431 cells that did not express CD25 were not lysed (Fig. 7B,C). The activity after coating with the scHLA-A2/scFv fusion was significantly improved compared to CTLs alone in the standard assay (Fig. 7A) (relative cytotoxicity of 140-180%, Fig. 7C,D, and E) indicating the potency of the effect. The activity in these assays of the covalent and non-covalent molecules was compared and was found to be indistinguishable (Fig. 7B,D, and E). To demonstrate the specificity of cov-scHLA-A2/aTac(scFv)-mediated CTL killing for the HLA-A2-restricted antigenic peptide, we used two CTL clones specific for the gp100 major T-cell epitopes 209 and 280. As shown in Fig. 7B-C, CD25-positive, HLA-A2-negative ATAC4 cells that were coated with 209 cov-scHLA-A2/aTac(scFv) were lysed by the 209-specific CTL clone R6C12 but not with R6C12 CTLs alone. Similar results were observed when these cells were tested with a 280 cov-scHLA-A2/scFv molecule and the JR1E2 CTL clone specific for the 280 peptide was used (Fig. 7C). ATAC4 cells coated with cov-scHLA-A2/αTac(scFv) with covalently linked 209 or 280 were not lysed when the 280-specific clone JR1E2 or 209-specific clone R6C12 was used respectively. Efficient cell lysis could also be observed with cov-scHLA-A2/scFv fusion that was generated with the EBV-derived peptide (Figure 7D). Thus, potent viral-specific CTLs can be targeted to kill tumor cells by coating with the fusion molecule. Similar results were observed with HLA-A2-negative ATL HUT102W leukemic cells that naturally express CD25 (Figure 7E). Target cells

were killed only when pre- incubated with the anti-Tac scFv-MHC, followed by the addition of the appropriate 209-specific R6C12 CTLs. The killing activity of the covalent scHLA-A2 and the non-covalent scHLA-A2 scFv fusion molecules were found to be quite similar, although the covalent scHLA-A2/scFv exhibited moderate, but reproducible, improved activity (Fig. 7E).

Note that the killing assays presented in Figure 7E were performed with a relatively low target:effector ratio, which may indicate the potent and sensitive ability of the scFv-MHC fusion molecule to induce CTL-mediated killing on target cells that express the natural CD25 target. To demonstrate the potency of the covalent scHLA-A2/scFv fusion molecules we performed a titration experiment on HUT102w leukemia cells with the covalent scHLA-A2/aTac(scFv) molecules carrying a covalently fused 209-peptide. As shown in figure 7F, a dose dependent effect was observed with an IC50 of ~50nM. These results demonstrate that the potentiation of CTL-mediated killing after coating target cells with cov-scHLA-A2/scFv fusion molecules was peptide and surface target antigen-specific.

DISCUSSION

Soluble MHC molecules with bound peptides are being used as reagents to study TCR-peptide-MHC interactions by either affinity measurements or directly visualizing antigen-specific T cells (42). In the latter case, multimerization of the MHC molecules overcomes the naturally low affinity of TCRs for MHC ligands, producing a higher avidity via multipoint binding. In both of these types of studies, the quality of the reagent depends on the complete occupancy of the MHC peptide binding groove with a single peptide. Although this has been achieved for both class I and class II MHC by loading "empty" MHC molecules in the presence of high concentrations of the peptide, the inherent instability of MHC molecules in the absence of a peptide can reduce the yield and, for certain MHC alleles and isotypes, result in denaturation without significant peptide loading. One alternate approach has been the covalent attachment of the peptide to the MHC molecule via a linker to the peptide (23-31). The structure of the peptide binding groove of class II MHC allows longer peptides to exit the groove at both the N-terminal and C-terminal ends. Therefore, the attachment of a linker to the C-terminal end was predicted to not interfere with peptide binding or T-cell recognition of the complex. This prediction was borne out by the crystal structures of numerous class II MHC molecules with covalently attached peptides (43-45).

For class I MHC, the C terminus of the peptide is intimately involved in MHC binding, and the groove is closed at both ends. It was not at first apparent how a linker could be attached to the peptide C terminus without disrupting peptide binding and/or interfering with T-cell recognition. However, several studies were performed with murine class I MHC-peptide complexes in which attaching the peptide to the N terminus of ß2m via a

flexible linker resulted in a high yield of fully assembled, soluble MHC class I molecules occupied by a single peptide and suitable for TCR interaction studies (23,24). Furthermore, crystal structures of soluble TCRs bound to MHC class I ligands (46,47) suggest that, depending on the exact length and position of the Vß CDR1 loop, room exists for the peptide linker attached to the peptide C terminus to exit the interface without disrupting ßTCR binding.

However, studies with human class I MHC were not reported. The strategy we used in this study was to generate a fully covalent HLA-A2-peptide molecule encoded by a single gene in which the peptide was attached to the N terminus of a single-chain ß2m-heavy chain HLA-A2 molecule. We have previously demonstrated that the scHLA-A2 is functional. We chose a linker length of 15 amino acids, based on crystal structures of MHC class I, predicting that the path from the peptide C terminus to the ß2m N terminus could be comfortably covered by a flexible linker of this length without interfering with TCR binding. Our experiments with three tumor or viral-derived peptides support this rationale, since this covalent ligand interacted specifically with T-cell lines and clones raised to HLA-A2 plus a free peptide. This interaction was demonstrated both by direct binding of a tetravalent cov-scHLA-A2 to specific CTLs and by the stimulation of the response of CTLs, as measured by IFNγ release.

Human MHC molecules with covalently linked peptides offer improved molecules, not only for molecular studies but also for generating new fusion molecules for immunotherapeutic approaches. Such approaches were recently introduced by us and others in which a human MHC molecule can be coupled or fused to a tumor-targeting antibody or its fragment in order to recruit T cells for specific killing of tumor cells (17-

22). Antibody-guided and tumor-specific targeting of class I MHC-peptide complexes onto tumor cells was shown to be an effective and efficient strategy to render HLA-A2-negative cells susceptible to lysis by the relevant HLA-A2-restricted CTLs. This new strategy of redirecting CTLs against tumor cells takes advantage of the use of recombinant antibody fragments that can localize on those malignant cells expressing a tumor marker, usually associated with the transformed phenotype (such as growth factor receptors and differentiation antigens), with a relatively high degree of specificity. The tumor-targeting recombinant antibody fragments consist of the Fv variable domains, which are the smallest functional modules of antibodies necessary to maintain antigen binding. This makes them especially useful for clinical applications, not only for generating the molecule described here but also for making other antibody fusion proteins, such as recombinant Fv immunotoxins or recombinant antibody-cytokine fusions (48,49), because their small size improves tumor penetration. In this approach, the antibody-targeting fragment is fused to a scHLA molecule, which can be folded efficiently and functionally around an HLA-A2-restricted peptide. Such molecules can be designed around the desired peptide specificity whether tumor-specific or with viral-derived peptide epitopes. The presence of tumor-specific CTL responses in patients may suggest that these may be efficient to target. However, recent studies have demonstrated that these tumor-specific CTLs are not always optimal, because they are present very seldom and only at very low frequencies, and even when they are present at high frequencies, they may not be functional or anergic (50). Thus, a more active and promising source of CTLs can be recruited from circulating lymphocytes directed against common and very immunogenic T cell epitopes such as those derived from viruses or

bacterial toxins, which can also elicit a good memory response (51). It has been shown that CTL precursors directed against influenza, EBV, and CMV epitopes (peptides) are maintained at high frequencies in the circulation of cancer patients as well as healthy individuals and that these CTLs are usually active and possess a memory phenotype (40,41,51).

In this study we took this approach a step further to produce potentially more stable and efficient molecules in which the antigenic peptide (tumor or viral) is covalently linked. A major advantage of this approach is the expected stability of the MHC-peptide complex in the circulation. Although the antigenic peptides selected for this approach are of relatively high affinity to the MHC, their stability within the complex is limited, and they dissociate from the MHC peptide binding groove.

Thus, the covalent attachment of the peptide may produce MHC-scFv fusions with markedly improved stability and thus better biological activity. The covalent scFV-MHC fusion molecules may be also produced more efficiently and with improved yields because the production process does not require the addition of an external HLA-A2-restricted peptide during the refolding.

We are currently assessing the biological activity of the cov-scHLA-A2/scFv molecules in *in vivo* studies of pharmacokinetics, stability, and antitumor activity.

This strategy is another step toward the development of a useful, effective approach to recruit active CTLs for killing tumor cells via cancer-specific Antibody-guided targeting of scMHC-peptide complexes. The recombinant scFv-MHC fusion molecules could represent an important addition to the existing immunotherapy approaches, bridging an antibody and T lymphocyte attack on cancer cells.

MATERIALS AND METHODS

Cell lines

A431, ATAC4 (epidermoid carcinoma), HUT102W, CRII-2 (leukemia, ATL), FM3D (melanoma), were maintained in RPMI 1640 + 10% FCS (FM3D were maintained in medium containing an addition of 1mM HEPES buffer). The ATAC4 cell line is a human epidermoid carcinoma A431 cell line stably transfected with the IL-2 receptor α subunit (p55, Tac, CD25) (32, 33). The transfected cells were maintained with 500μg/ml G418 (Gibco-BRL).

Plasmid constructions

The scHLA-A2 molecule was constructed as previously described by linking human β2-microglobulin with the three extracellular domains of the HLA-A2 gene (34). To construct the scHLA-A2 with covalently-bound peptides, we fused the peptide and a $(Gly_4\text{-}Ser)_3$ linker to the scHLA-A2 by a PCR overlap extension reaction with the primers: (**G9-209M**: 5'GGAAGCGTTGGCGCATATGATCATGGACCAGGTTCCGTTCTCTG TTGGCGGAGGAGGGTCCGGTGGCGGAGGTTCAGGAGGCGGTGGATCGATCCAGCGTACTCCA AAG-3', **G9280V**:GGAAGCGTTGGCGCATATGTATCTGGAACCGGGTCCGGTTACCGTTG GCGGAGGAGGGTCCGGTGGCGGAGGTTCAGGAGGCGGTGGATCGATCCAGCGTACTCCAAAG -3', **EBV(280-288)**:5' GGAAGCGTTGGCGCATATGGGTCTGTGCACCCTGGTT GCTATGC TGGGCGGAGGAGGGTCCGGTGGCGGAGGTTCAGGAGGCGGTGGATCGATCCAGCGTACTCCA AAG**MART(MelanA)**:5'GGAAGCGTTGGCCATATGATCATGGACCAGGTTCCGTTCTCTGTTGGC GGAGGA GGGTCCGGTGGCGGAGGTTCAGGAGGCGGTGGATCGATCCAGCGTACTCCAAAG-3', **BirA-EcoRI**: 5' –GCAGTAAGGAATTCTTA TC AGCGGTGATTCCA-3')

To generate the cov-scHLA-A2/aTac(scFv) expression constructs, we fused the relevant antigenic peptide to the N-terminus of the scHLA-A2/aTac scFv molecule using a single-step PCR overlap extension reaction incorporating the appropriate primers as described above. The PCR product was subcloned into the TA cloning system for sequencing and

subsequently to a T7-promotor-based expression vector using the *NdeI* and *EcoRI* restriction sites.

Expression, refolding, and purification of cov-scHLA-A2 and cov-scHLA-A2/aTac(scFv) fusion molecules

The cov-scHLA-A2 and cov-scHLA-A2/aTac(scFv) fusion molecules were expressed in *E. coli* BL21 (λDE3) cells (Novagen, Madison, WI). Inclusion bodies were purified by cell disruption with 0.2 mg/ml of lysozyme followed by the addition of 2.5% Triton X-100 and 0.5M NaCl. The pellets of inclusion bodies were collected by centrifugation (13000 RPM, 60 min at 4°C) and washed 3 times with 50mM Tris buffer, pH 7.4, containing 20mM EDTA. The isolated and purified inclusion bodies were solubilized in 6M Guanidine HCl, pH 7.4, followed by reduction with 65mM DTE. Solubilized and reduced inclusion bodies were refolded by a 1:100 dilution into a redox-shuffling buffer system containing 0.1M Tris, 0.5M Arginine, and 0.09mM Oxidized Glutathione, pH 10.0 . scHLA-A2-peptide complexes and antibody Fv-fusion with non-covalent bound peptides were refolded using the same protocol except that a 10-fold molar excess of the antigenic peptide was added to the refolding solution. After having been refolded, the protein was dialyzed against 100 mM Urea, 20 mM Tris, pH 7.4, followed by purification of the soluble cov scHLA-A2/aTac scFv complexes by ion-exchange chromatography on a Q-Sepharose column (7.5mm I.D x60cm) (Pharmacia), applying a salt (NaCl) gradient. Peak fractions containing the cov-scHLA-A2/aTac scFv fusion were then subjected to buffer exchange to PBS.

ELISA

Immunoplates (Falcon) were coated with 10 μg/ml of purified bacterially produced recombinant CD25 (O/N at 4°C) . The plates were blocked with PBS containing 2% skim milk and then incubated with various concentrations of cov-scHLA-A2/aTac(scFv)-peptide or scHLA-A2/aTac(scFv)-peptide complexes (90 min at RT). Binding was detected using the anti-HLA conformational dependent antibody W6/32 (60 min, RT, 10μg/ml). The reaction was developed using anti-mouse IgG-peroxidase. Human anti-Tac antibody was used as a positive control, followed by anti-human peroxidase.

Flow Cytometry

The cells were incubated with cov-scHLA-A2/scFv fusion molecules (60 min at 4°C in 100μl, 10 μg/ml), washed and incubated with the anti-HLA-A2 MAb BB7.2 (60 min at 4°C, 10μg/ml). Anti-mouse FITC served as a secondary antibody. Tetramer staining of the CTLs was performed with cov-scHLA-A2 multimers labeled with streptavidin PE (20ng/μl, 4°C, 1 hour). Human anti-Tac (10μg/ml) was used as a positive control to determine the expression of the p55 antigen, followed by incubation with anti-human FITC-labeled antibody. The cells were subsequently washed and then analyzed by a Beckman FACScaliber flow cytometer.

CTL clones and stimulation

CTL clones specific for the melanoma gp100-derived peptides were kindly provided by Drs. Steven Rosenberg and Mark Dudley, Surgery Branch, National Cancer Institute, NIH. These CTL clones were generated by cloning from bulk cultures of PBMCs from patients receiving peptide immunizations (35). R6C12 and JR1E2 CTLs were maintained

in RPMI 1640 + 20% AIM-V medium +10% heat-inactivated human serum + 1mM HEPES + Pyruvate (gibco 1:100) MEM non-essential amino acides (gibco 1:100). CTL clones were expanded by incubation with irradiated melanoma FM3D cells (as a source of antigen) and the EBV-transformed JY cells (B-lymphoblasts as antigen-presenting cells). The stimulation mixture also contained the OKT3 antibody (30 ng/ml) and 50 IU/ml of IL-2 and IL-4.

CTL lines specific for the EBV BMLF1 protein-derived peptide 280-288 were kindly provided by Dr. Dietmar Zehn (Charitee, Berlin). These CTLs were expanded from a healthy HLA-A2 positive donor and were maintained in AIMV medium + 8.9% FCS + 50uM 2-mercaptoethanol + Penicillin/streptomycin $1x10^5$ U/L.

Stimulation assay and Interferon γ release

EBV peptide-specific T cells ($1x10^5$) were incubated in the presence of fluide phase tetrameric scHLA-A2 at the concentrations indicated generated with covalent (Cov) or non-covalent (scHLA-A2) peptides for 24 hrs at 37°C. Controls were cells incubated with termaric scHLA-A2 containing covalent or non-covalent gp100-derived peptides 209 and 280. Activation was measured by the release of Interferon γ to the culture supernatant. Interferon γ was measured in culture supernatants by an ELISA assay kit (IFNγ-FLEXIA, BioSource Inc, Camarillo, CA).

Cytotoxicity assays

Cytotoxicity was determined by ^{35}S-methionine release assays. Target cells were cultured in 96-well plates ($2-5x10^3$ cells per well) in RMPI+10% FCS, washed and incubated with

methionine and serum-free medium for 2 hr followed by incubation (O/N) with 15 μCi/ml of ^{35}S-methionine (NEN). After 3hr of incubation with cov-scHLA-A2/scFv fusion molecules (at 37°C, 40 nM), effector CTL cells were added at a target:effector ratio as indicated, and incubated for 8-12 hr at 37°C. Following incubation, ^{35}S-methionine release from target cells was measured in a 50μl sample of the culture supernatant. All assays were performed in triplicate. The percentage lysis was calculated relative to the activity of CTLs on FM3D melanoma cells. With EBV-specific CTLs lysis was calculated directly: ([experimental release – spontaneous release]/[maximum release – spontaneous release]) x100. Spontaneous release was measured as ^{35}S-methionine released from target cells in the absence of effector cells, and maximum release was measured as ^{35}S-methionine released from target cells lysed by 0.1M NaOH.

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Figure Legends:

Figure 1: Schematic and design of scHL-A2 and covalent scHLA-A2/scFv fusion proteins.

(A) In the single-chain HLA-A2 gene, the human β-2m is fused to the three extracellular domains of HLA-A2 via a flexible 15-amino acid-long linker ($[Gly_4\text{-}Ser]_3$).

(B) The same peptide linker is used to connect the C-terminus of an antigenic HLA-A2-restricted peptide to the N-terminus of the scHLA gene. The antgenic peptides used in this study are melanoma differentiation antigen gp100-derived peptides G9-209M (209) and G9-280V (280) in which anchor positions 2 and 9, respectively were mutated to increase binding affinity to HLA-A2 (52), melanoma derived-$MART1_{27\text{-}35}$ peptide and the EBV-derived peptide $GLC_{280\text{-}288}$. In A and B, the BirA sequence tag for site specific biotinylation was introduced at the C-terminus of the HLA-A2 gene.

(C) A scFv antibody fragment gene, composed of the antibody V_H and V_L variable domains linked together with a $(Gly_4\text{-}Ser)_3$ flexible linker, was fused to the C-terminus of the covalent peptide-scHLA-A2 gene or to the scHLA-A2 gene with a non-covalent peptide as a control (D). The HLA-A2/scFv fusions do not contain a C-terminal BirA tag.

Figure 2: Expression and purification of scHLA-A2 with covalently linked peptides.

ScHLA-A2 with 4 covalently linked peptides were expressed in *E. coli* and accumulated in inclusion bodies. (A) SDS-PAGE analysis of isolated inclusion bodies. (B) SDS-PAGE analysis of scHLA-A2 with covelently linked gp100-derived peptide 209 and EBV-derived peptide after ion-exchange purification. (C) SDS-PAGE analysis of a

scHLA-A2 with covalently linked peptide 209 fused to the anti-Tac scFv antibody fragment after purification on ion-exchange chromatography.

Figure 3: Characterization of scHLA-A2 with covalently linked peptides.
(A) Conformation of scHLA-A2 generated with covalently linked peptides. ELISA assay of scHLA-A2 complexes with non-covalent and covalent (cov) bound peptides. Complexes at 10μg/ml were immobilized through a BSA-biotin-Sterptavidin bridge to immunoplates and the reactivity of the conformation-sensitive MAb w6/32 (10μg/ml) was used to prove its correct conformation. Detection was with HRP-labeled secondary antibody. A non-specific control of reactivity with BSA was subtracted from experimental values. (B) Activation of T cells with covalent scHLA-A2 complexes. EBV peptide-specific T cells (10^5 cells/well) were incubated in the presence of fluide phase tetrameric scHLA-A2 generated with covalent (Cov) or non-covalent (scHLA-A2) peptides at the indicated concentrations for 24 hrs at 37°C. Controls were cells incubated with termaric scHLA-A2 containing covalent or non-covalent gp100-derived peptides 209 and 280. Activation was measured by the release of Interferon γ to the culture supernatant by an IFNγ ELISA assay.

Figure 4: Binding of scHLA-A2 with covalently linked peptides to T cells.
EBV $GLC_{280-288}$ –specific CTLs (A) or melanoma differentiation antigen gp100-specific CTL clones R6C12 (B) and R1E2 (C) were reacted with *in vitro* refolded purified scHLA-A2 tetramers containing the covalent EBV, 209 epitope recognized by R6C12

CTLs and 280 peptide recognized by R1E2 CTLs. For a comparison, CTLs were stained with scHLA-A2 tetramers generated by refolding with the appropriate peptide (non-covalent). In A and B, CTLs were stained with FITC-anti-CD8 and with PE-labeled covalent and non-covalent tetramer. In C, CTLs were stained with PE-labeled tetramer alone. Controls are scHLA-A2 with covalently linked irrelevant peptide.

Figure 5: Characterization of covalent scHLA-A2/scFv fusion

(A) Binding of covalent scHLA-A2/aTac(scFv) to CD25. CD25 was immobilized onto immunoplates and the dose-dependent binding of covalent scHLA-A2/aTac(scFv) with three differnet covalently linked peptides was monitored by the binding of the confomation-sensitive MAb W6/32. Binding was detected with a HRP-labeled secondary antibody. (B) Comparison of the binding of non-covalent and covalent scHLA-A2/aTac(scFv) to CD25. Binding was monitored with MAb W6/32. Backgound is with w6/32 and secondary antibody alone, without scHLA-A2/scFv fusion.

C. Titration of the binding of covalent (o) and non-covalent (Δ) scHLA-A2/aTac(scFv) to immoblized CD25. Binding was detected with MAb W6/32 and secondary HRP-labeled antibody.

Figure 6: Binding of covalent scHL-A2/scFv fusion to target cells.

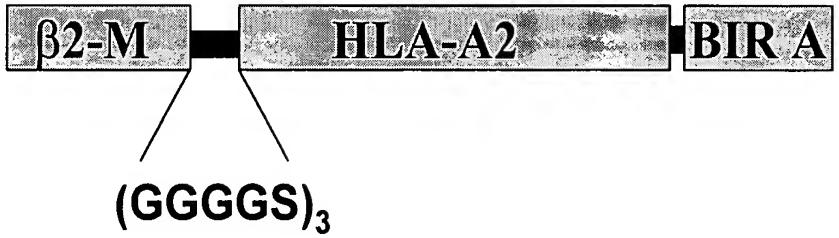
Flow cytometry analysis of the binding of covalent scHLA-A2/aTac(scFv) fusion to antigen-positive HLA-A2-negative cells. Binding of anti-Tac Mab to control parental A431 cells (A), A431 cells transfected with CD25 (ATAC4) (B), and HUT102W leukemic cells naturally expressing CD25 (C). D-K, the binding of anti-HLA-A2 MAb

BB7.2 to ATAC4 (D,F,H,J), HUT102W (E,I) and control A431 (G,K) cells before and after incubation with covalent or non-covalent scHLA-A2/aTac(scFv) containing the melanoma-derived gp100-derived peptides 209 and 280. In D and H, the binding of covalent and non-covalent scHLA-A2/scFv is compared. A431 cells are CD25 and HLA-A2-negative (C,G, and K). Binding of MAbs BB7.2 and anti-Tac were monitored with FITC-labeled secondary antibody.

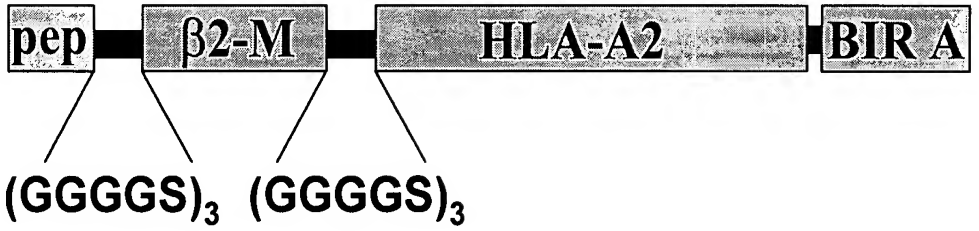
Figure 7: Potentiation of CTL-mediated lysis of HLA-A2-negative tumor cells by covalent scHLA-A2/scFv fusion.

(A) Activity of gp100-derived 209 and 280-specific CTLs on FM3D melanoma cells. (B) A431 and CD25-transfected ATAC4 HLA-A2$^-$ cells were preincubated or not with covalent and non-covalent scHLA-A2/aTac(scFv) generated with peptide 209 or 280 as a control followed by incubation with the 209-specific CTL, R6C12. The control ATAC4 cells were incubated with CTL alone. (C) A431 or CD25-transfected ATAC4 cells were preincubated with covalent scHLA-A2/aTac(scFv) fusion generated with EBV GLC280-288-derived peptide followed by EBV peptide-specific CTLs. Controls are ATAC4 cells incubated with CTLs alone. (D) CD25-transfected ATAC4 HLA-A2$^-$ cells were preincubated with covalent and non-covalent scHLA-A2/aTac(scFv) fusion generated with gp100-derived peptides 209 and 280 followed by incubation with peptide 280-specific CTL clone JR1E2. (E) HUT102w leukemic cells coated or not with covalent and non-covalent scHLA-A2/aTac(scFv) fusion generated with peptides 209 or 280. (F) Titration of covalent scHLA-A2/aTac(scFv) fusion on HUT102w leukemic cells.

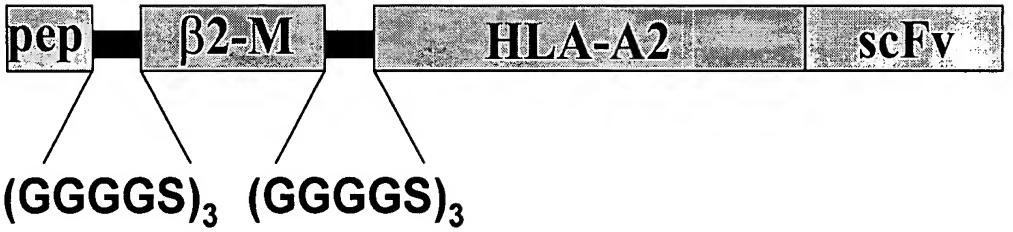
A. scHLA-A2.1



B. cov-scHLA-A2.1



C. cov-scHLA-A2.1/scFv



D. scHLA-A2.1/scFv

Figure 1

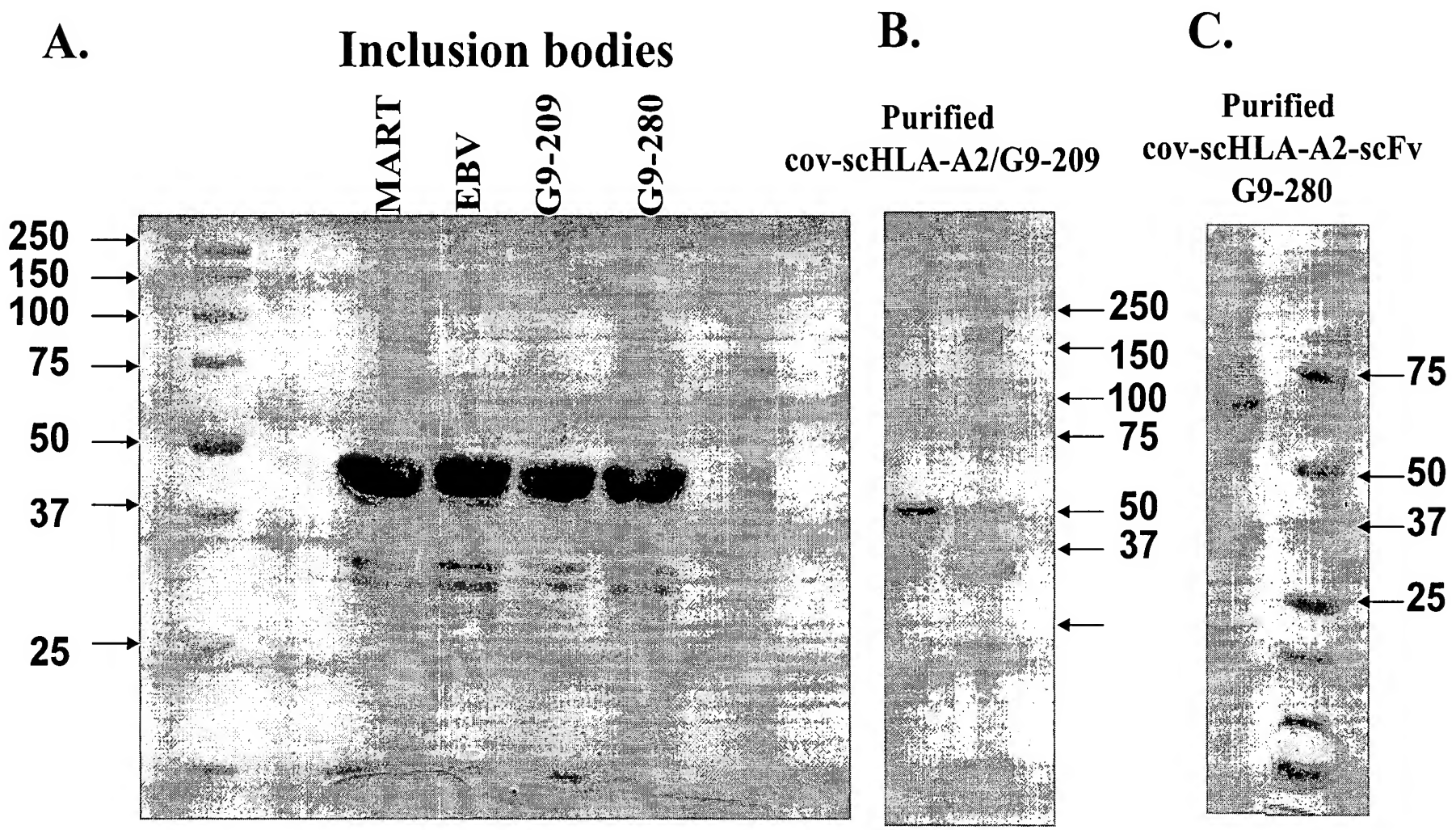
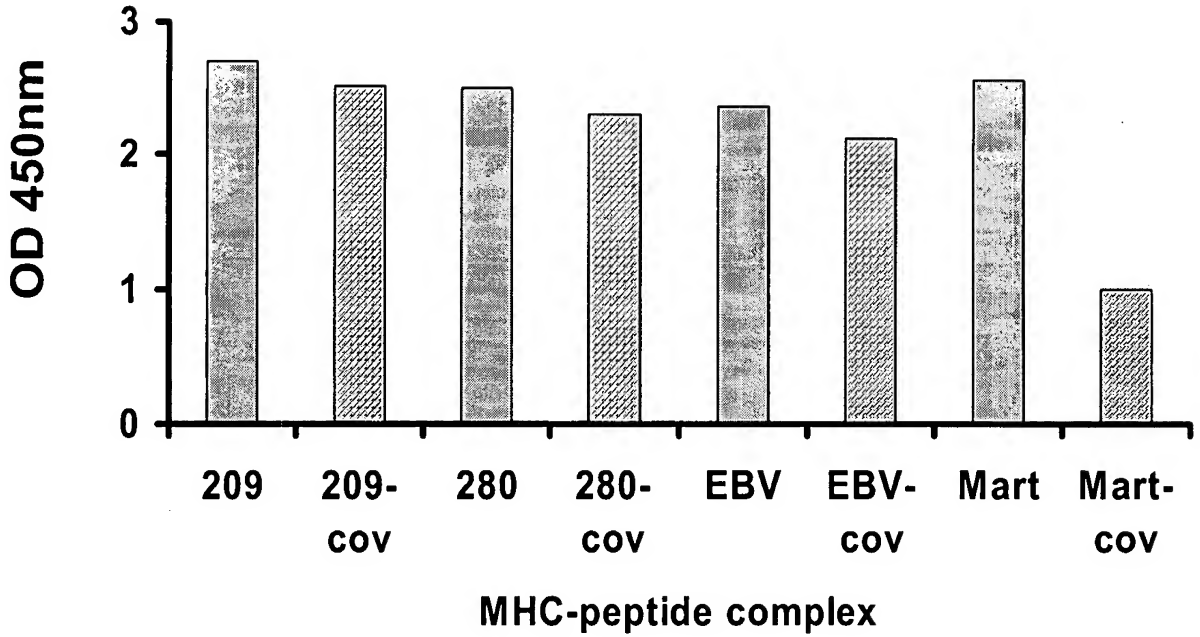

A.
Inclusion bodies
MART
EBV
G9-209
G9-280
250
150
100
75
50
37
25
B.
Purified
cov-scHLA-A2/G9-209
250
150
100
75
50
37
C.
Purified
cov-scHLA-A2-scFv
G9-280
75
50
37
25

Figure 2

A


OD 450nm
3
2
1
0
209
209-cov
280
280-cov
EBV
EBV-cov
Mart
Mart-cov
MHC-peptide complex

B

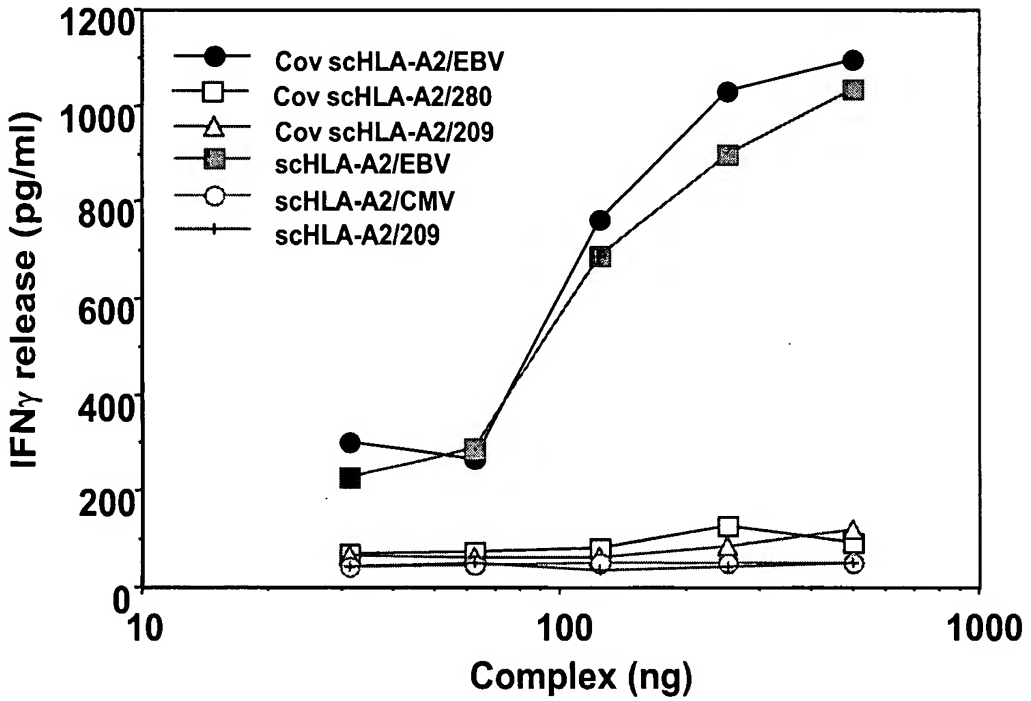

IFNγ release (pg/ml)
1200
1000
800
600
400
200
0
Cov scHLA-A2/EBV
Cov scHLA-A2/280
Cov scHLA-A2/209
scHLA-A2/EBV
scHLA-A2/CMV
scHLA-A2/209
10
100
1000
Complex (ng)

Figure 3

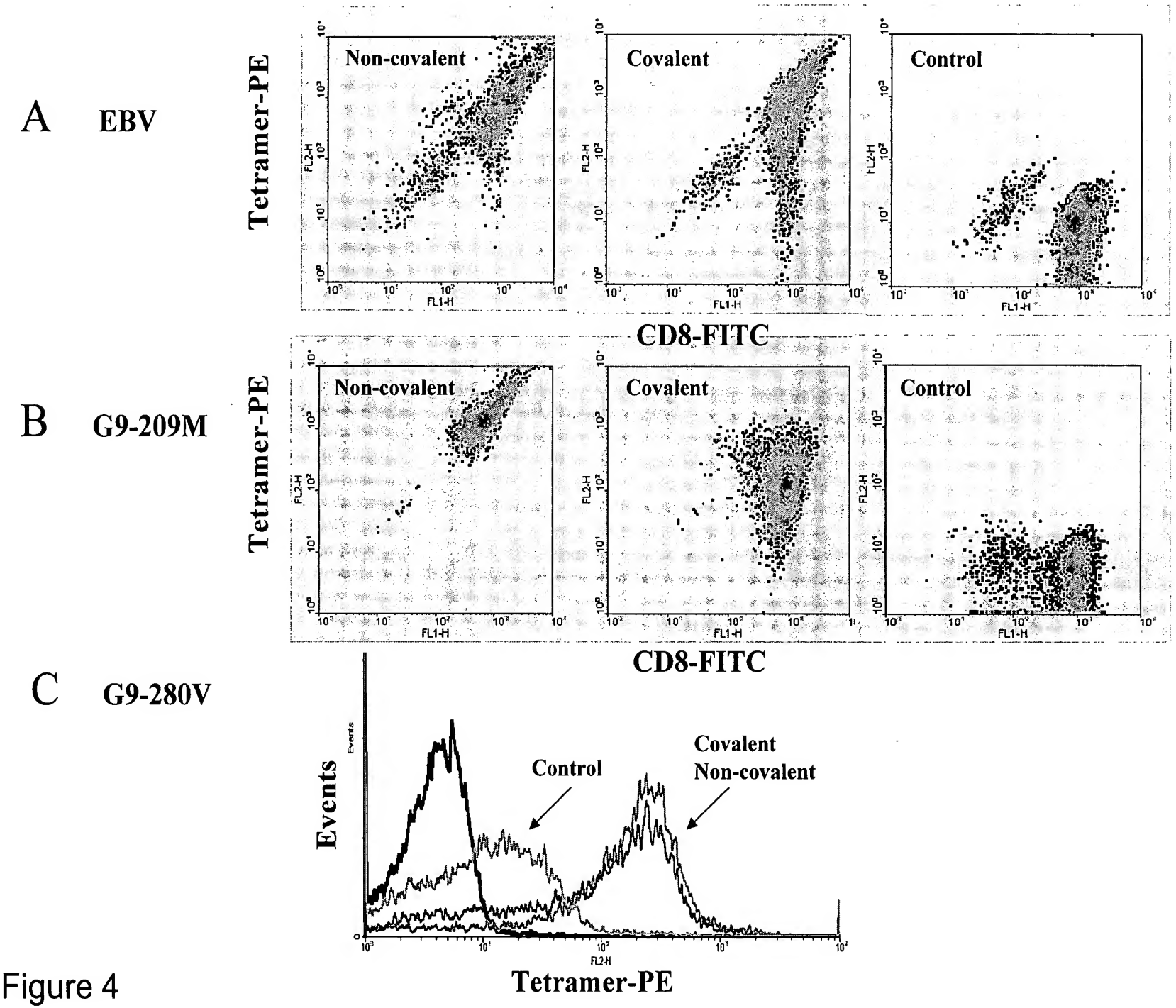
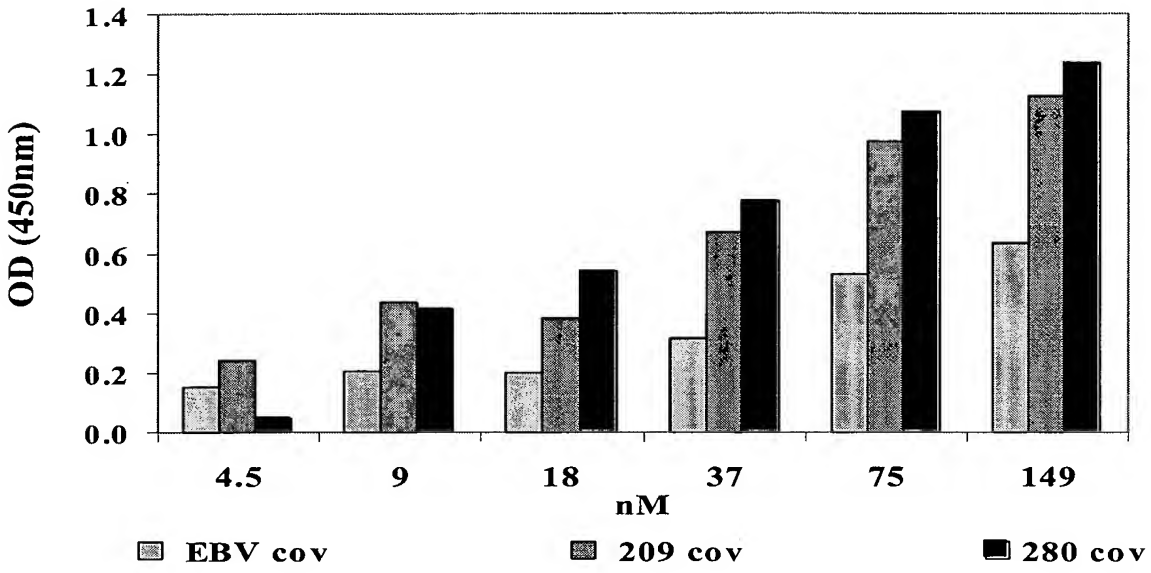
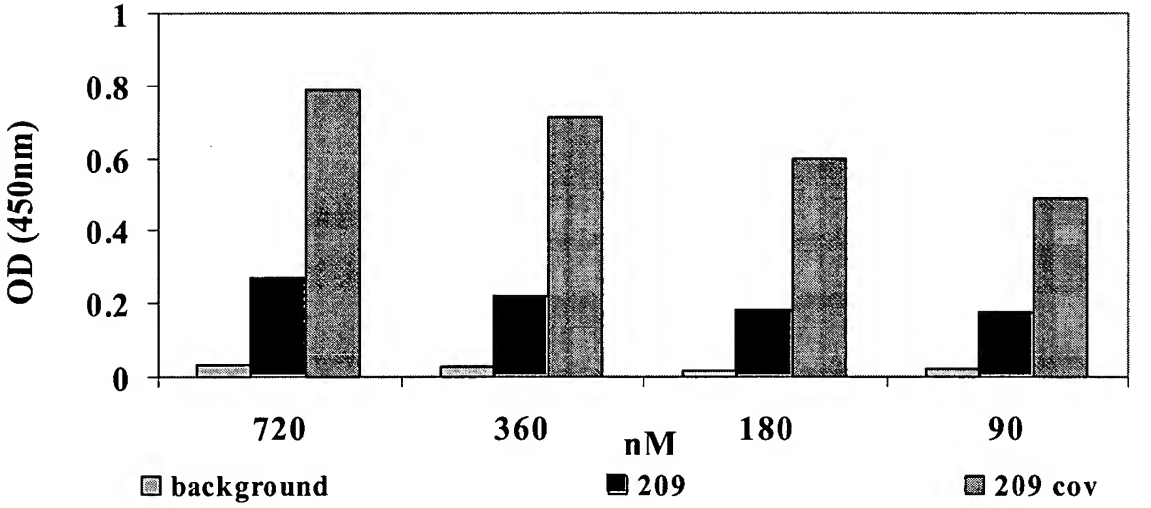

A EBV
Tetramer-PE
Non-covalent
Covalent
Control
FL2-H
FL1-H
CD8-FITC
B G9-209M
Tetramer-PE
Non-covalent
Covalent
Control
FL2-H
FL1-H
CD8-FITC
C G9-280V
Events
Control
Covalent
Non-covalent
FL2-H
Tetramer-PE

Figure 4

A


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD (450nm)
4.5
9
18
37
75
149
nM
EBV cov
209 cov
280 cov

B


1
0.8
0.6
0.4
0.2
0
OD (450nm)
720
360
180
90
nM
background
209
209 cov

C

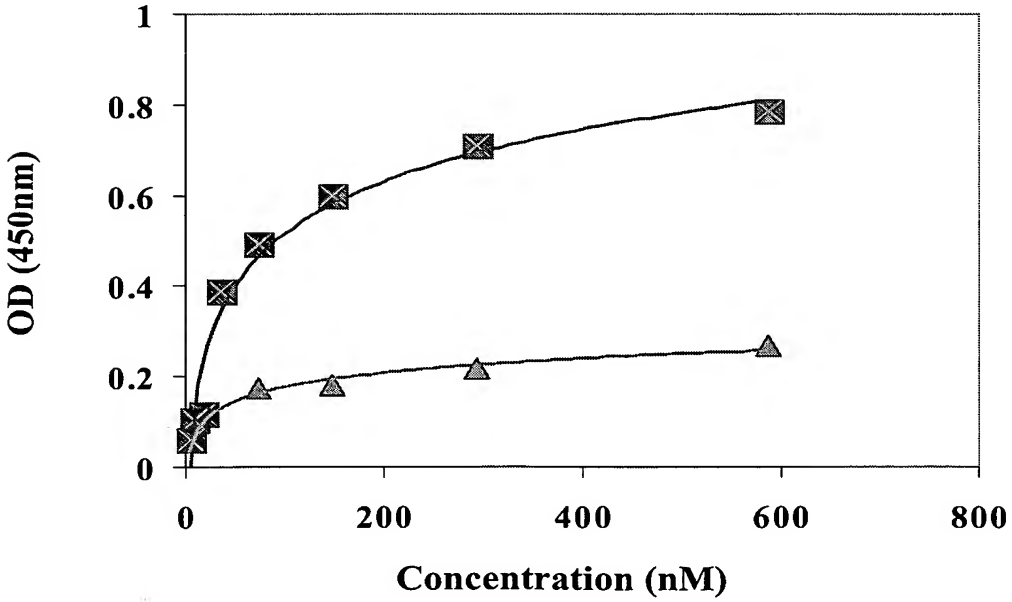

1
0.8
0.6
0.4
0.2
0
OD (450nm)
0
200
400
600
800
Concentration (nM)

Figure 5

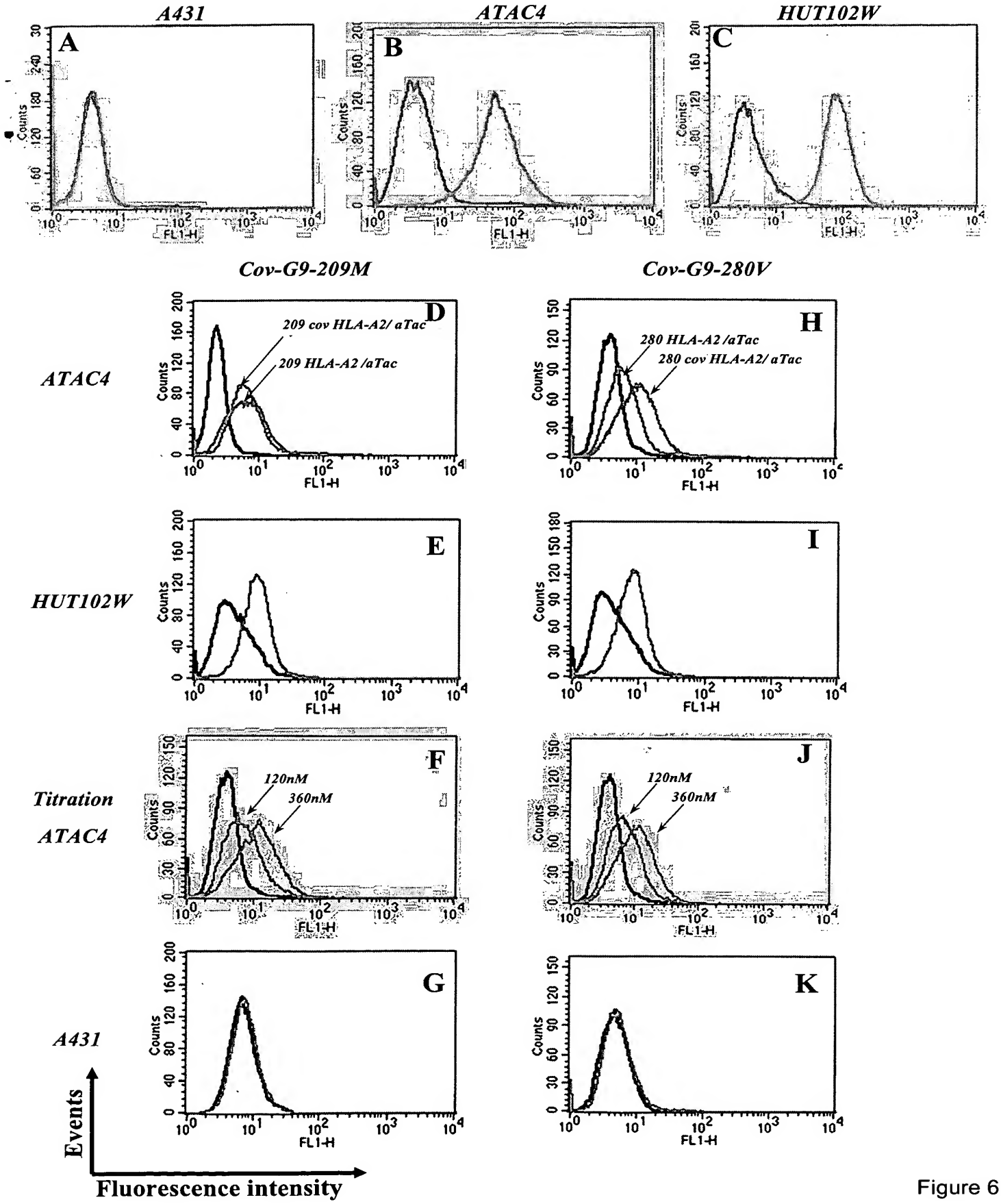
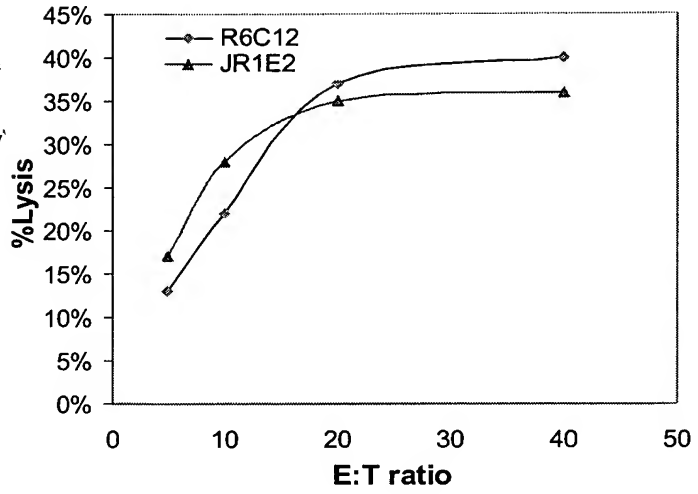
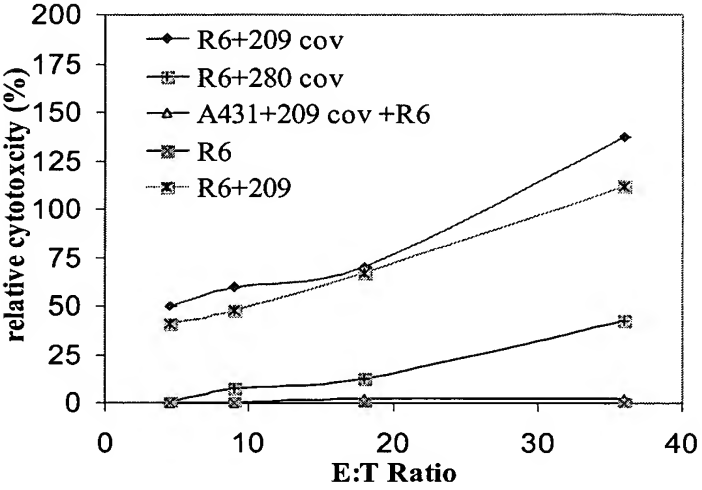

A431
ATAC4
HUT102W
A
B
C
Cov-G9-209M
Cov-G9-280V
ATAC4
D
209 cov HLA-A2/ aTac
209 HLA-A2 /aTac
H
280 HLA-A2 /aTac
280 cov HLA-A2/ aTac
HUT102W
E
I
Titration
ATAC4
F
120nM
360nM
J
120nM
360nM
A431
G
K
Counts
FL1-H
Events
Fluorescence intensity

Figure 6

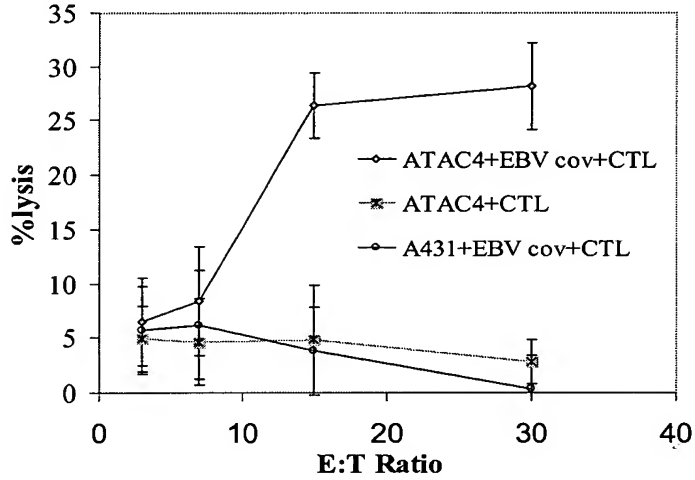
A. FM3D



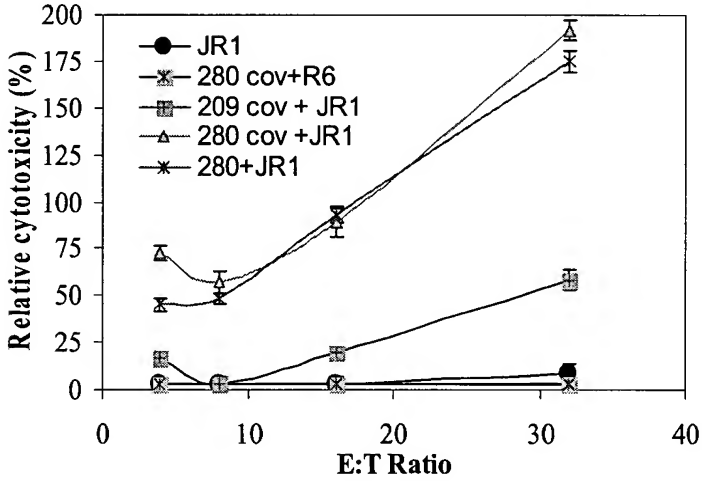
B. 209/209cov



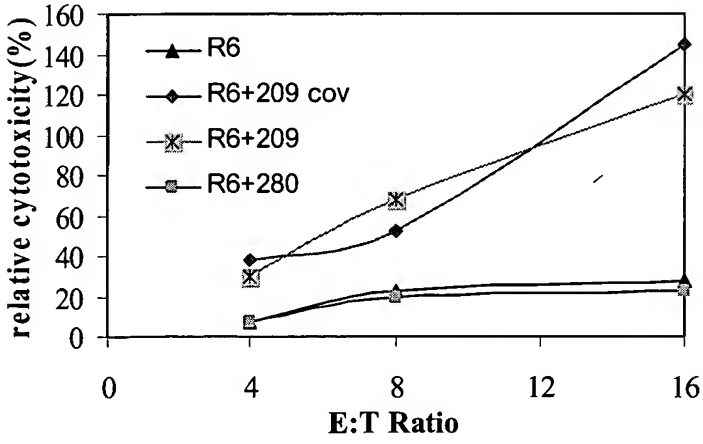
C. EBVcov



D.280/280cov



E. HUT102 – 209/209cov



F.209cov titration

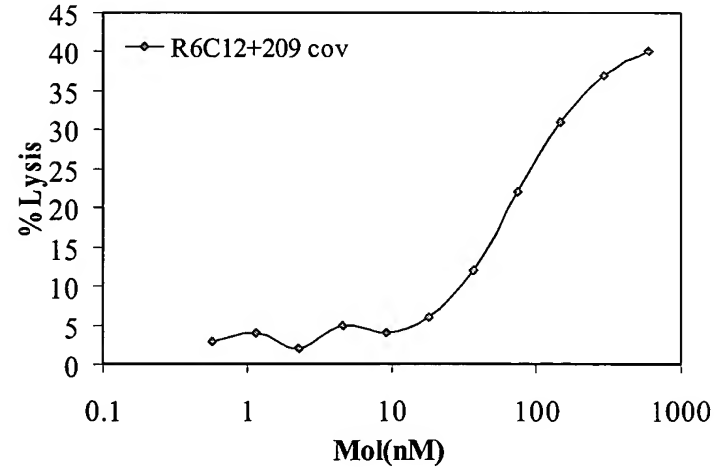


Fig 7